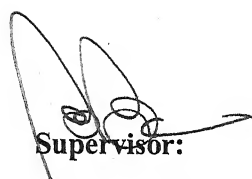


**Thesis for the Degree of Doctor of Philosophy in  
Biotechnology  
Bundelkhand University, Jhansi**

**Title: “Proteomic Analysis For The Identification Of Human  
Macrophage Molecule/s Involved In Mycobacterial  
Killing: *Sphingosine 1-Phosphate as a novel enhancer of  
phospholipase-D mediated antimycobacterial activity*”.**



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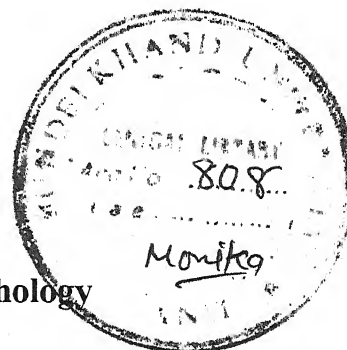
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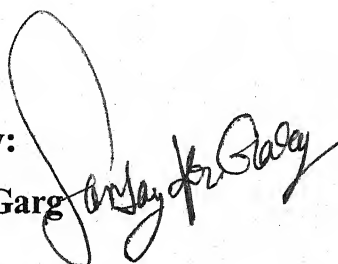
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*Research is an evolving concept. Any endeavor, in this regard, is challenging as well as exhilarating. It implies the testing of our nerves. It brings to light our patience, vigor, and dedication. Every result arrived at is a modest beginning for a higher goal. In this context my thesis in the same spirit, is just a step in the ladder. It is a drop in the ocean.*

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## Abbreviations

1, 25-dihydroxy vitamin D <sub>3</sub>	-	1, 25-(OH) <sub>2</sub> D <sub>3</sub>
Acquired immunodeficiency syndrome	-	AIDS
ADP-ribosylation factor	-	ARF
Antigen presenting cells	-	APCs
Alkaline phosphates	-	AP
Arabinogalacton	-	AG
Bacillus Calmette Guirin	-	BCG
Bovine serum albumin	-	BSA
Cell-mediated immunity	-	CMI
Colony forming unit	-	CFU
Complement receptors	-	CR
Delayed- typed hypersensitivity	-	DTH
Dendritic cells	-	DCs
Deoxyribonucleic acid	-	DNA
Diacylglycerol	-	DAG
Directly observed therapy short-course	-	DOTS
Distilled water	-	dH <sub>2</sub> O
Double distilled water	-	ddH <sub>2</sub> O
<i>Escherichia coli</i>	-	<i>E. coli</i>
Ethylene diamine tetra acetic acid	-	EDTA
Enzyme linked immunosorbent assay	-	ELISA
Endothelial differentiation gene receptors	-	EDGR
Ethambutol	-	EMB
Gamma-interferon	-	IFN- $\gamma$
G-protein-coupled receptors	-	GPCR
High density lipoprotein	-	HDL
Major histocompatibilty complex	-	MHC

Hors Reddish Peroxides	-	HRPO
Human immunodeficiency virus	-	HIV
Human leukocyte antigen	-	HLA
Immunoglobulin receptors	-	FcRs
Inositol 1,4,5-trisphosphate	-	IP <sub>3</sub>
Interleukin	-	IL
Isoniazid	-	INH
Kilo Dalton	-	kDa
Lipoarabinomannans	-	LAM
Lipopolysacchrides	-	LPS
Lysosomal associated membrane protein	-	LAMP
Macrophage mannose receptor	-	MMR
Matrix assisted laser desorption ionization-Time of Flight	-	MALDI-TOF
Monocyte derived macrophages	-	MDM
Multi-drug resistant	-	MDR
<i>Mycobacterium smegmatis</i>	-	Msm
<i>Mycobacterium tuberculosis</i>	-	MTB
Nitric oxide synthase	-	NOS2
Nitric oxide	-	NO
p-aminobenzoic acid	-	PABA
Para-aminosalicylic acid	-	PAS
Peptide finger printing	-	PMF
Phosphate buffer saline	-	PBS
Phosphate buffer saline with Tween-20	-	PBST
phenolicglycolipid I	-	PGL-I
Phosphatidic acid	-	PA
Phosphatidyl choline	-	PC
Phosphatidylinositol 4,5-bisphosphate	-	PIP <sub>2</sub>

Phosphatidylinositol	-	PI
Phospholipase A <sub>2</sub>	-	PLA <sub>2</sub>
Phospholipase D	-	PLD
Phospholipase C	-	PLC
Phosphotidylethanol	-	Pet-OH
Platelet-derived growth factor	-	PDGF
Protein kinase C	-	PKC
Pyrazinamide	-	PZA
Reactive nitrogen intermediates	-	RNI
Reactive oxygen intermediates	-	ROI
Rifampicin	-	RFP
Ribonucleic acid	-	RNA
Sodium dodesyl sulphate	-	SDS
Sphingosine 1-phosphate	-	S1P
Sphingosine kinase	-	SPHK
Streptomycin	-	SM
<i>t</i> ryptophan <i>a</i> spartate-containing <i>co</i> at	-	TACO
Tris acetate EDTA	-	TAE
Tris EDTA	-	TE
Tetra methyl benzedine	-	TMB
Toll-like receptor	-	TLR
Transferrin receptor	-	TFR
Transporter of antigen processing	-	TAP
Tuberculosis	-	TB
Tumor necrosis factor-alpha	-	TNF- $\alpha$
Two-dimensional gel electrophoresis	-	2-DE
World Health Organization	-	WHO

## Introduction

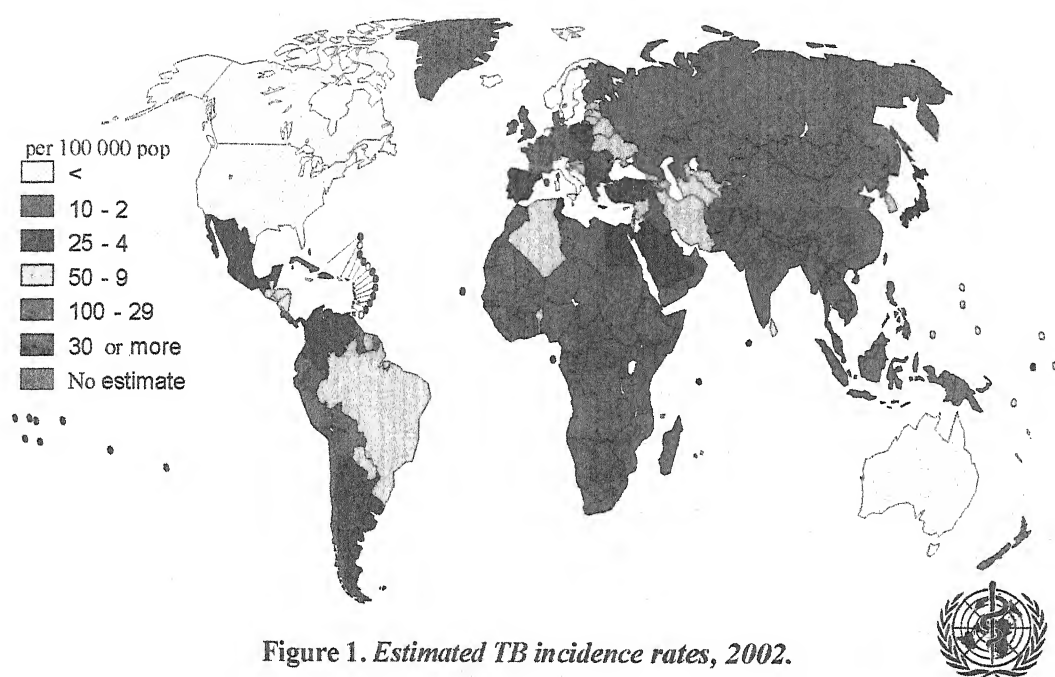
### 1. Tuberculosis and global burden

Tuberculosis (often called TB) is an infectious disease and is primarily a disease of the respiratory system, which usually attacks the lungs, but can attack almost any part of the body. Transmission of TB usually occurs by inhalation of infectious droplet nuclei containing one to three *M. tuberculosis* (MTB) bacteria, or ingestion of contaminated material, usually milk. When person with pulmonary TB cough, laugh, sneeze, sing, or even talk, the MTB may be spread into the air. These MTB-containing droplet nuclei can remain suspended in the air for several hours. Infection will occur if inhalation by another person, results in the organism(s) reaching the alveolus of the lungs.

TB is a disease found mostly in poor socioeconomic and underprivileged groups. Thus, the disease usually occurs in areas with over-crowded populations such as slums, correctional facilities, refugee camps and shelters for the homeless. Moreover, people whose work may expose them to TB, such as health care workers, are also at higher risk of MTB infection and TB disease. However, infection with HIV, treatment with corticosteroids, aging, and alcohol or drug abuse increase the potential for reactivation of latent TB [45]. The classic TB signs- cough, expectoration, hemoptysis, wasting of body were well recognized. The patient suffering with active disease, coughs frequently with thick sputum and sometimes contains blood. His breathing is like a flute and skin is cold with hot feet. He sweats greatly and heart is much disturbed. When the disease is extremely grave, he suffers from diarrhea.

TB is the world's second commonest cause of death from infectious disease after HIV/AIDS. The World Health Organization (WHO) estimates that at least one-third of the world population (or 2 billion people) is infected with MTB, leading to 2 million deaths each year, which constitutes about 26% of the avoidable adult deaths in the developing world. Every year, 8 million people become sick with TB, of whom 95% are in the developing countries. Major parts

of the two continents (Asia particularly South and South-East, and Africa particularly Sub-Saharan region) account for 3/4 of the worlds annual TB cases, depicting clearly the connection of this disease with poverty. Some industrialized countries have recently seen outbreak of this health problem mainly due to HIV infection and/or emergence of multi drug resistant (MDR) strains. The global TB case load appears to be growing slowly, and without urgent action at national and international level, TB will claim about 70 million more lives between now and the year 2020, and there will be about 200 million new cases of active disease [153].



TB among HIV-infected people has become an epidemic within epidemic. With the emergence of AIDS, TB and HIV infection have been intimately connected. HIV infection is the single strongest risk factor for the progression of TB from primary as well as latent to active disease, while MTB infection accelerates the probability of HIV progression in co-infected individuals [123]. Hence, in countries that have a high prevalence of HIV infection, the incidence of TB is increasing. In fact, in 1990, TB and HIV co-infection constituted 4% of all TB cases, but by the year 2001, co-infection reached a dramatic level of nearly 1 out of every 7 cases of TB (15%). In Asia and Africa, about 60% and 40% of AIDS patients, respectively, have TB [141].



Surprisingly, of the 15 million people estimated to be co-infected with HIV and MTB, nearly 12 million (76%) live in Sub-Saharan Africa [154]. The situation is likely to worsen in the near future, if urgent remedies are not carried out [140].

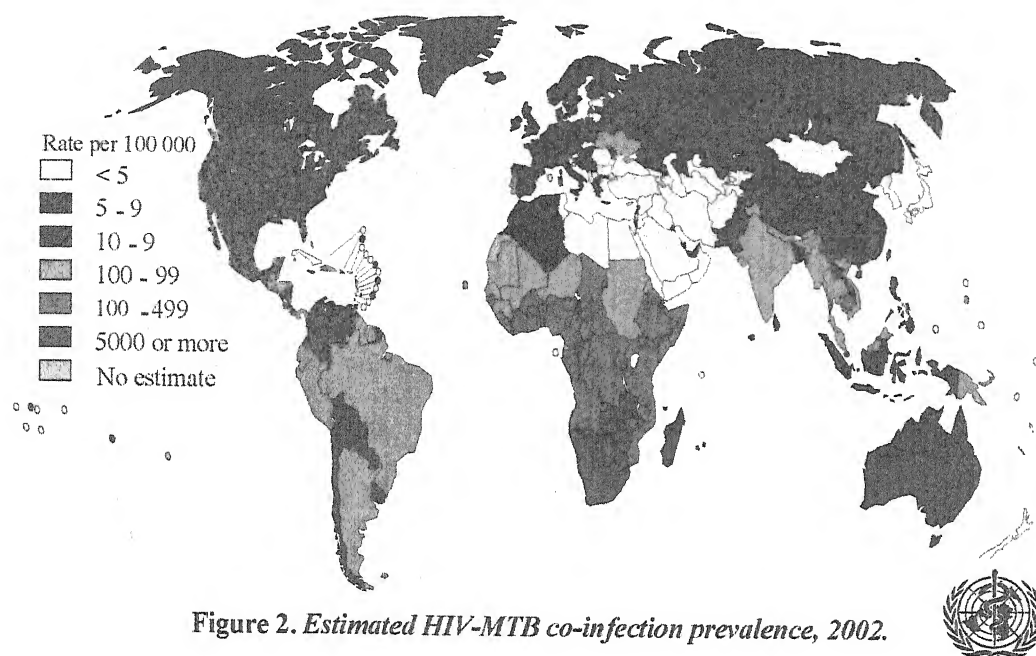


Figure 2. Estimated HIV-MTB co-infection prevalence, 2002.



### 1.1 History of tuberculosis

The establishment of TB in humans is still not clear, but certain reports suggest that TB probably occurred as an endemic disease among animals long before it affected humans [134]. *Mycobacterium bovis* had been causing TB in the animal kingdom long before invading humanity and probably was the most likely cause of human TB. In the Paleolithic period, people lived as wanderers, did not settle in villages and did not congregate in large groups. However, after the development of primitive agricultural techniques and subsequent domestication of cattle between 8000-4000 BC, there is evidence of human infection by *M. bovis* likely through milk ingestion [21]. This coincides with archeological evidence of spinal TB (Potts disease) at 5000-1000 BC. Thus, *M. bovis* was the likely pathogen in human TB until ~1000 BC and after 1000 BC, widespread pulmonary TB emerged. In fact, MTB probably is an evolved, specialized form of *M. bovis*, developed initially among milk-drinking Indo-Europeans who then spread the

disease during their migration into Western Europe and Eurasia. By the 1st millennium BC, MTB, the etiologic agent of modern TB had spread throughout the known world.

TB has had many aliases throughout history. The earliest written evidence of pulmonary TB was from the library of the Assyrian king Assurbanipal (668-626 BC). It was existed even in Vedic age i.e. nearly 5000 years ago and was mentioned in Rig-Veda as "**Rajayakshma**". Two famous Indian physicians Charak and Susruta have also given detailed description of the disease. From the time of Hippocrates, TB was known as "**phthisis**", a term derived from the Greek for "**wasting away**". The swollen glands of the neck were termed "**scrofula**", and because in medieval times newly crowned kings of England and France were believed to have powers to heal TB with their touch so it was also called as "**The Kings Evil**". TB of the skin was known as "**lupus vulgaris**" and that of the bones was termed as "**Potts disease**" with characteristic vertebral fusion and deformity of the spine. In fact, this had enabled historians to establish the existence of TB in mummies dating from 2000 to 4000 B.C. The most familiar term for TB, at least to our ancestors was "**consumption**", which means to consume or wear away. Whatever mask it wore, TB was responsible for one-fifth to one quarter of the deaths with some socially and scientifically important loss such as Frederick Chopin (1849), Laennec (1826), John Keats (1821), Robert Louis Stevenson (1894), Max Lurie (1920), John Harvard, James Arthur Gass, and the well known Bronte Family (1813-1820) during the first half of the 19th century.

## 1.2 *Mycobacterium tuberculosis*



*M. tuberculosis* (MTB) is the etiologic agent of present TB in humans which transmit mostly from person to person via aerosol. MTB is a straight or slightly curved bacillus, non-motile, non-encapsulated with 2-4  $\mu\text{m}$  in length and 0.2-0.5  $\mu\text{m}$  in width and

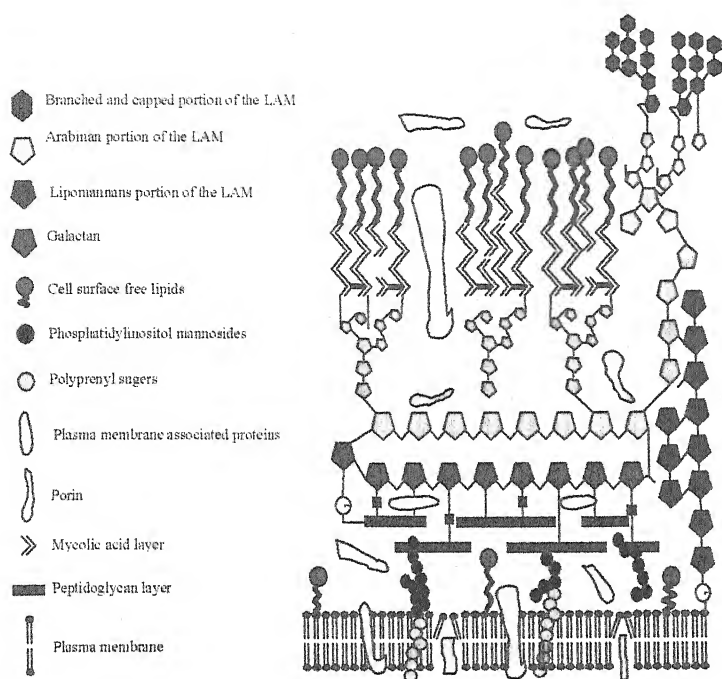
does not form spores. At taxonomic point of view, MTB is classified in the class- *Schizomiceti*, order-*Actinomycetes*, family-*Mycobacteriaceae*, and genus-*Mycobacterium*. Other pathogens belonging to the mycobacterium genus include- *M. bovis*, which causes bovine as well as human

TB, *-M. avium*, *-M. intracellualrlae* which are known as opportunistic pathogen and cause a TB-like disease especially in AIDS patients and *-M. leprae*, the causative agents of leprosy. Other species such as *M. smegmatis*, *M. terrae* are known non-pathogenic environmental mycobacterium.

MTB is an obligate aerobic organism. For this reason, in the classic case of TB, the MTB complexes (*M. tuberculosis*, *M. africanum*, *M. bovis*, and *M. microti*) are always found in the well-aerated upper lobes of the lungs. The bacterium is a facultative intracellular parasite, usually of macrophages, and has a slow generation time (15-20 hours), that may contribute to its virulence. MTB colonies are small and buff colored when grown either on Middle brook's agar based or Lowenstein-Jenson egg based medium. Chains of cells in smears made from *in vitro*-grown colonies often form distinctive serpentine cords. This observation was first made by Middle brook (1947) who associated cord factor with virulent strains of the bacterium

MTB is not classified as either Gram-positive or Gram-negative because it does not have the chemical characteristics of either, although the bacteria do contain peptidoglycan (murein) in their cell wall. If a Gram stain is performed on MTB, it stains very weakly Gram-positive or not at all, which is referred to as "ghosts". *Mycobacterium* species along with members of related genus *Nocarida*, are classified as acid-fast bacteria due to their impermeability by certain dyes and stains. One acid-fast staining method for MTB is the Ziehl-Neelsen stain. In this method, the MTB smear is heat fixed, stained with carbol-fuchsin (a pink dye), and decolorized with acid-alcohol. Finally, the smear is counter stain with methylene-blue or certain other dyes. Acid fast bacilli appear pink in a contrasting background.

The cell wall of mycobacteria has several unique features which distinguish it from all other prokaryotes. Roberts Koch first pointed out the unusual cell wall of MTB and its importance in mycobacterial physiology followed by several others [12, 29, 96]. The cell wall consists of a plasma membrane surrounded by a lipid and carbohydrate rich wall, which in turn is enriched by a capsule of polysaccharides, proteins and lipids. The plasma membranes appears to be typical of all bacterial membranes and partly resembles a Gram-positive wall with a peptidoglycan layer



**Figure 3.** *Schematic representation of MTB cell envelope.*

composed of N-acetyl- $\beta$ -D-glucosaminyl- (1-4)-N-acetyl-muramic acid units [29]. The cell wall core is chemically composed of three covalently linked principle macromolecules: highly cross linked peptidoglycan covalently attached via a linker unit (L-Rha-D-GlcNAc-P) to a linear galactofuran of arabinogalacton (AG), and several

strands of a highly branched arabinofuran of AG, which in turn attached to mycolic acids, a hydrophobic wax like character [12, 29, 96]. Mycolic acids represent a major constituent of the mycobacterial cell wall complex, which provides the first line of defense against potentially lethal intracellular environmental conditions.

Recently the MTB genome has been sequenced, with following important features [23].

- First major pathogen to be sequenced.
- 4,411,522 bps.
- 3,924 open reading frames.
- GC content of 65.6%.
- 70% of the genes can be identified at this stage, the remainder are unique and encode proteins with unknown functions.
- 59 % of genes are transcribed in the same direction as chromosomal replication.

## **2. Pathogenesis of tuberculosis**

TB is spread by airborne droplet nuclei, which are particles of 1-5  $\mu\text{m}$  in the diameter that contain MTB. Because of their small size, the particles can remain airborne for minutes to hours after expectoration by people with pulmonary or laryngeal TB during coughing, sneezing, singing, or talking [45]. The infectious droplet nuclei are inhaled and lodge in the alveoli in the distal airways. MTB is then taken up by alveolar macrophages, initiating a cascade of events that result in either successful containment of the infection or progression to active disease.

The development of pulmonary TB from its onset to its various clinical manifestations may be pictured as a series of battles between host and invader. (i) The inhaled bacilli may multiply, or it may be eliminated by alveolar macrophages before any lesion is produced, (ii) Small caseous lesions (a few millimeters in the diameters) may progress or may heal or stabilize before they are detectable by radiograph, (iii) Larger caseous lesions may grow locally and shed bacilli into the blood and lymph, or they may heal or stabilize, (iv) Alternatively, caseous lesions may liquefy and introduce bacilli and their products into the bronchial tree, making arrest of the disease more difficult. In general, each successive battle is won by the host with increasing difficulty. Once the bacilli survive and the first small caseous tuberculous lesion is established, all subsequent battles occur in a host capable of both tissue damaging and macrophage-activating immune response. The former causes caseous necrosis if high local concentrations of the tuberculin-like bacillary products are present. Whereas the latter, often called cell-mediated immunity (CMI) or acquired cellular resistance, causes an accumulation of activated macrophages around the caseous center of the lesion. However, the balance between the macrophage-activating and tissue-damaging immune responses throughout the course of the disease determines whether the disease will progress or regress, and if it does progress, what form it will take.

### **2.1 Tissue-damaging and macrophage-activating immune response: dual mechanisms that control bacillary multiplication**

Both the tissue-damaging and macrophage-activating immune processes are complex, each with



many interactions and checks and balances. The tissue-damaging response is often produced during delayed-typed hypersensitivity (DTH) reactions to the tuberculin-like products of the bacillus, while in macrophages activating response, specific antigens locally stimulate T lymphocytes to produce lymphokines that attract and activate macrophages and lymphocytes. This tissue damaging hypersensitivity process kills non activated macrophages within which the bacillus is multiplying [30], whereas, the macrophages activating response, activates macrophages to kill and digest the bacilli they ingest. The killing of such bacilli-laden macrophages by the tissue - damaging immune response immediately stops this logarithmic multiplication of the bacillus, enabling the host to survive at the expense of some of its own tissues loss. Various inflammatory mediators, e.g., clotting factors, eicosanoids, cytokines, hydrolytic enzymes, and reactive oxygen and nitrogen intermediates, are frequently involved in one or both processes.

Conclusively, the tissue- damaging response produces caseous necrosis within which the bacilli are inhibited extracellularly and the macrophage-activating response produces activated macrophages within which the bacilli are killed intracellularly. They are two distinct immune responses for inhibiting the progression of the disease. For the successful management of TB, there is a real need to study the interplay between these two immune responses and alter the ratio between them, so that the host can control bacillary growth with minimal tissue destruction [31].

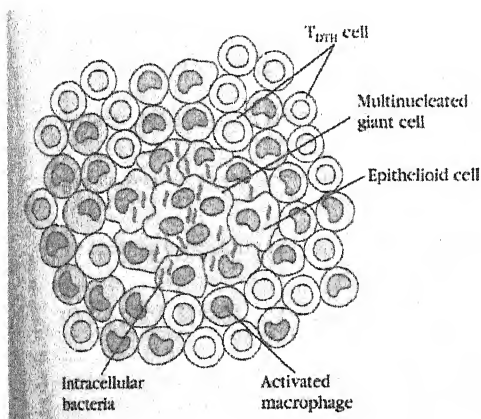
## **2.2 Stages in the pathogenesis of tuberculosis**

Max B. Lurie has established the different following stages of human TB on the basis of his experiments on susceptible and resistant strains of rabbits [85].

**Stage I: Onset** - The first stage begins following the inhalation of the tubercle bacilli into an alveolus. There, an alveolar macrophages ingest the bacillus and often destroys it. This destruction depends on the inherent microbicidal power of the alveolar macrophages and the genetic and phenotypic virulence of the ingested bacillus. A virulent bacillus in relatively weak alveolar macrophages seems to be able to multiply and initiate the disease, but a weak bacillus in

strong activated alveolar macrophages seems to be readily destroyed or inhibited before bacillary replication can occur. Alveolar macrophages are cells that have been already and non-specifically activated by a variety of inhaled particles, or by ingestion of occasional extravagated erythrocytes, and/or possibly by general stimulation of the mononuclear phagocytes system. Therefore, the microbicidal ability of the alveolar macrophages exists before tubercle bacilli are inhaled. CMI (in which antigens stimulate specific T cells to activate macrophages) is not involved in the immediate destruction of inhaled tubercle bacilli [85] because the only particles small enough to reach the alveolar spaces contain no more than three bacilli, and few bacilli do not possess enough antigen to elicit either a primary or a secondary immune response.

**Stage II: Symbiosis** - If the original alveolar macrophages fails to destroy or inhibit the inhaled bacilli, the bacilli multiply until that macrophages bursts, resulting in release of bacilli, cell debris, and variety of chemotactic factors of host origin, which attracts the other neighbor alveolar macrophages and non-activated macrophages (i.e., monocytes emigrating from blood stream) to ingest its bacillary load. In this time, immature macrophages from the circulation become completely responsible for the fate of the early lesion and development of symbiotic relation [85], in which neither the macrophages nor the bacilli injure each other, because these



immature macrophages have not yet activated, so they cannot inhibit or destroy the bacilli, and the bacilli cannot injure the macrophages because the host has not yet developed tuberculin type hypersensitivity. With the time, more and more macrophages and more and more bacilli accumulate in the lesion.

Figure 4. A typical tuberculous granulomatous structure.

**Stage III: Initial caseous necrosis** - The third stage of disease begins when the logarithmic bacillary multiplication stops. At that time (2-3 weeks after the inhalation of the bacilli), the host becomes tuberculin positive and the lesions undergo caseous necrosis in their

centers, where the bacilli containing non-activated macrophages are killed by tissue damaging immune response. Thus, host by destroying bacilli containing non-activated macrophages, eliminates the intracellular favorable environment for such uncontrolled growth of bacilli that would otherwise be fatal [14]. The tubercle bacilli however, can remain alive in this solid caseous material, but it apparently can not multiply because of the anoxic conditions, reduced pH, and the presence of inhibitory fatty acids [106].

**Stage IVa or IVb: Interplay between tissue damaging and macrophage-activating immune response** - The size of the caseous necrosis is depending on the susceptibility / resistivity of host. In susceptible host, tubercle bacilli escaped from the edge of the caseous center are ingested by around non-activated incompetent macrophages. In such macrophages, bacilli again find favorable intracellular environment in which to multiply until the tissue damaging immune response kills these new bacilli-laden incompetent macrophages and the area of caseous necrosis enlarges. Therefore, the susceptible host (including infant and immunosuppressed adults) must continue to use the tissue-damaging immune response (necrotizing DTH) to stop the intracellularly bacillary multiplication in the expense of organ (lung) destruction. Finally, the bacilli spread by the lymphatic and hematogenous routes to other sites, where the tissue destructions continue. In case of resistant host caseous center remains small, because bacilli escaping from the edge of necrosis center are engulf by surrounded highly activated competent macrophages. In such macrophages bacilli cannot multiply and are eventually destroyed. Such effective activated macrophages are produced by bacillary antigens expanded specific T-cells populations and their lymphokines (such as  $\text{INF } \gamma$ ). If the caseous center remains solid, inspissates and does not liquefy, the disease will be arrested by this strong CMI process, because further tissue destruction does not occur. This scenario occurs in healthy immunocompetant humans beings who show positive tuberculin reactions and yet no clinical and often even no X-ray evidence of the disease [30, 85].



**Stage V:** Unfortunately, in some resistant host as well as in immunocompetent adults, due to strong CMI, progression of disease may occur. Such progression is caused by liquefaction and cavity formation [86]. Liquefied caseous foci and cavities do not occur in susceptible host, probably due to weak CMI bacilli do not contained in to solid caseous and hence already disseminated. The factors that cause liquefaction are largely unknown, but in experimental animals, liquefaction is associated with high levels of tuberculin reactivity [157] and elevated hydrolytic enzymes such as proteinases, nucleases, and lipases [33]. A subtle change in the nature of the tissue damaging pathways may be involved [157]. The breakdown products of the caseous material are osmotically active. It absorbs the water from the surrounding tissue, and an excellent culture medium for tubercle bacilli is created. In fact, studies suggested that liquefied material is an excellent growth material for the tubercle bacilli. At this stage, for the first time during the course of disease the bacillus multiplies extracellularly, often reaching tremendous numbers. Because the host is now highly sensitive to the tuberculin-like products of the bacillus, this large antigenic load is quite toxic to the tissues. Macrophages do not survive in liquefied caseous material, possibly after entering into the liquefied caseum they are killed by toxic fatty acids originating from the host cells and/or the bacilli [61]. As a result of tissue destruction the walls of nearby bronchi often become necrotic and rupture, forming a cavity. Then, the bacilli and the liquefied caseous material are discharged into the airways and reach other parts of the lung and the outside environment, by coughing.

The walls of the cavities consist of an external zone of collagen, that forms capsule of the cavity (which in humans is occasionally hyalinized), and an internal zone of caseous (often liquefying), where the high oxygen contents from ambient air nurtures the growth of bacilli [32]. In humans, between the external zones and internal zones, there is a zone of granulation tissue rich in capillaries, granulocytes, macrophages, lymphocytes, and fibroblasts [32]. Thickness of all three zones is depends on the stage of the disease. With the newly formed cavities, the internal caseous zone is thickest, and with older, still active cavities the external capsule is thickest. Around the capsule, usually between the pleura and the cavity, an area of atelectasis is often present. This atelectasis area may prevent perforation into the pleural spaces. Erosion of

incompletely thrombosed vessels in the intermediate zone leads to hemorrhage into the wall of the cavity. From there, blood may pool and thereby give rise to some hemoptysis. Massive hemoptysis, however, is usually due to the leakage or rupture of a fully patent blood vessel located in the wall of the cavity or traversing lumens. At present, no therapeutic agents are available to prevent liquefaction, but appropriate antimicrobial therapy can eliminate the most of the bacilli from the cavity. Consequently, inflammation then decreases, and much of all of the necrotic contents drains through the still patent bronchocavity junction, are resulting in the arrest of the disease.

### **3. Innate immunity and macrophages**

Healthy individuals protect themselves against microbe's invaders by means of many different mechanisms. Some of these protective mechanisms comprise innate (also called natural, or naive) immunity, which are the first line of defense mechanisms against microbe's invader. The characteristics of innate immunity are 1) rapidity 2) non-specificity (limited capacity to distinguish one from another) and 3) no immunologic memory. The principal components of innate immunity are 1) anatomical or physical barriers (such as skin, epithelia, intestinal movement); 2) secretory molecules (such as blood proteins, including members of the complement and anti-microbial substances produced at epithelial surfaces); and 3) cellular components (phagocytic cells; neutrophils, macrophages and other leukocytes, such as natural killer cells). Innate immunity provides the early lines of defense against microbes. However in the other hand, the pathogenicity of microbes is, in part, related to their ability to resist the mechanisms of innate immunity.

In contrast to innate immunity, adaptive immunity are highly evolved defense mechanisms, that are stimulated by exposure to infectious agents, and increase in magnitude and defensive capabilities with each successive exposure to a particular microbe. Because of its capacity to distinguish between different microbes, adaptive immunity is also called as specific immunity. The components of specific immunity are lymphocytes and their products, such as antibodies. Specific immune responses are classified into two types, based on the components of the

immune system that mediate the response. 1) Humoral immunity, that is largely mediated by antibodies, produced by specific cell, called B lymphocytes; 2) Cell-mediated immunity, that is mediated by cells called T lymphocytes. Humoral immunity is the principal defense mechanism against extracellular microbes and their secreted toxins because antibodies can bind to these and assist in their elimination. In contrast, intracellular microbes, such as viruses and some bacteria, survive and proliferate inside phagocytes and other host cells, where they are inaccessible to circulating antibodies. Hence, defense against such infections are due to cell-mediated immunity, which functions -by promoting the destructions of microbes residing in phagocytes, or -by lysing the infected cells.

The mechanisms of both innate and specific immune response make up an integrated system of host defense in which numerous cells and molecules function cooperatively. The cells of the immune system are normally present - as circulating cells in the blood and lymph, - as anatomically defined collections in lymphoid organs, and - as scattered cells in virtually all tissues except the central nervous system. The anatomical organization of these cells and their ability to circulate and exchange among the blood, lymph, and tissues are of critical importance for the generation of immune responses. Normal blood cells, as a part of immune component consists neutrophils, eosinophils, basophils, lymphocytes, and monocytes. Fundamental component of cellular innate immunity are mononuclear phagocytes (monocytes, macrophages), which constitutes the second major cell population of the innate immune system. This system consists of cells that have a common lineage whose primary function is phagocytosis. All the cells of the mononuclear phagocytes system originate from bone marrow stromal cell and, then pass through the following differentiation steps: stem cell- committed stem cell - monoblast - promonocyte - monocyte (bone marrow). After the maturation and subsequent activation, these cells can achieve varied morphologic forms. The first cell type that enters the peripheral blood after leaving the marrow is incompletely differentiated cell and is called the monocyte. Monocytes are 10-15  $\mu\text{m}$  in diameter, having bean-shaped nuclei and finely granular cytoplasm containing lysosomes, phagocytic vacuoles, and cytoskeleton filaments. The blood monocytes are young cells that already possess migratory, chemotactic, pinocytic and phagocytic activities,

as well as receptors for IgG Fc-domains (Fc- $\gamma$ R) and iC3b complement. Under migration into tissues, monocytes undergo further differentiation (at least one day) to become multifunctional tissue macrophages, which are 15-20  $\mu$ m diameters with abundant cytoplasm (containing lysosomes, phagocytic vacuoles, and cytoskeleton filaments), several pseudopodia like structure, and often fused to form multinucleated giant cells. Moreover, they are recognized by their morphology, by ability to adhere on glass/plastic surface, by phagocytic property and by presence CD14 (mac-2) marker. Different anatomical sites of macrophages display a diversity of phenotypes, capabilities, and functions (histiocytes in connective tissue, Kupffer's cells in liver, alveolar macrophages in lung, free and fixed macrophages in lymph nodes and in spleen, fixed macrophages in bone marrow, pleural and peritoneal macrophages in serous fluids, histiocytes in skin, Langerhans's cell in intestine, mesengial phagocytes in kidney and microglial cells in brain) Principal function of mononuclear phagocytes in innate and adaptive immunity is the phagocytosis of foreign invaders and relaying of other effector mechanism. At the site of infection, phagocytes can attach to foreign invaders (such as bacteria) via a -number of receptors for bacterial components (scavenger receptor, LPS receptor, mannose receptor, Toll-like receptors, etc.) or -host protein that act as opsonins (protein which aid phagocytosis such as fibronectin, complement and IgG antibody). This attachment triggers internalization of the organisms through phagosome formation with subsequent activation of respiratory burst (hexose monophosphate shunt), and phagosome lysosome fusion. At this stage the phagocytosed organisms can be killed by either of three effectors mechanisms. 1) Oxygen-dependent myeloperoxidase-independent killing (by products of respiratory burst such as superoxide anion, singlet oxygen, hydroxyl ion and hydrogen peroxide); 2) Oxygen-dependent myeloperoxidase-dependent killing (by halogenation of bacterial proteins catalyzed by meyloperoxidase); and, 3) Phagolysome fusion (by lysosomal antibacterial substances such as lactoferrin, cationic proteins, lysozyme, defensins, proteases etc.). A defect in any of these pathways, for instance, due to absence of NADPH oxidase, myeloperoxidase, may predispose an individual to increased susceptibility to infections. Moreover, macrophages produce various cytokines which play a distinct role in the initiation and control of immune system. In this context, type 2 nitric oxide

synthase (iNOS or NOS2) was originally described as an enzyme that is expressed in activated macrophages, generates nitric oxide (NO) from the amino acid l-arginine pathway, and thereby contributes to the control of replication or killing of intracellular microbial pathogens.

Mononuclear phagocytes are important participant in the bidirectional interactions between innate and specific immunity. Macrophages that respond to microbes as a reaction of innate immunity also function to trigger microbes-specific lymphocytes responses. Conversely, effector lymphocytes and their products enhance the anti-microbial functions of macrophages.

To this regard, 1) macrophages act as an antigen presenting cells (APCs), by displaying foreign antigen on their surface to let them recognized by antigen-specific T lymphocytes. Macrophages also produce cytokines (IL-12) that stimulate T cell proliferation and differentiation. In addition, activated macrophages express surface proteins, called co-stimulators that augment T cell responses at sites of infection and inflammation. Thus, macrophages functions as accessory cells in lymphocyte activation.

Moreover, 2) in the effector phase of certain cell-mediated immune response, antigen-stimulated T cells secrete cytokines that activate macrophages. Such activated macrophages are more efficient at performing microbicidal functions than are un-stimulated cells and are thus better able to destroy phagocytosed microbes. Thus, macrophages are among the principal effector cells of cell-mediated immunity.

Finally, 3) during the effector phase of humoral immune response, foreign antigens, such as microbes, become coated, or opsonized, by antibody molecules and complement proteins. Because macrophages express surface receptors for antibodies and for certain complement proteins, they bind and phagocytosed opsonized particles much more avidly than uncoated particles. Thus, macrophages participate in the elimination of foreign antigens by humoral immune response.

#### 4. Macrophages and its anti-mycobacterial response

The mononuclear phagocyte constitutes a potent antimicrobial component of both innate and cell-mediated immunity. The precise mechanisms by which these cells mediate killing or inhibition of bacterial pathogens are not clearly understood. Upon entry, intracellular fate of bacilli is depending on its virulence state and macrophage activation state. However, it can multiply within resting macrophages, or can be killed / inhibited by activated macrophages. It is well established that murine macrophages possess anti-mycobacterial function in tissue culture systems. When activated by supernatants of immunologically stimulated lymphocytes, macrophages exhibited various degrees of anti-mycobacterial activity. Hydrogen peroxide ( $H_2O_2$ ), one of the reactive oxygen intermediates (ROI) generated by macrophages via the oxidative burst, was the first identified effector molecule that mediated mycobacteriocidal effects of mononuclear phagocytes [146]. The significance of ROI in host defense against MTB remains controversial. Later, gamma-interferon ( $IFN-\gamma$ ) was found to be the key endogenous activating agent that triggers the anti-mycobacterial effects of murine macrophages [114]. Tumor necrosis factor-alpha ( $TNF-\alpha$ ), although ineffective when used alone, synergizes with  $IFN-\gamma$  to induce anti-mycobacterial effects of murine macrophages *in vitro* [44]. A major effector mechanism responsible for the anti-mycobacterial activity of  $IFN-\gamma$  and  $TNF-\alpha$  is induction of the production of nitric oxide and related reactive nitrogen intermediates (RNI) by macrophages via the action of the inducible form of nitric oxide synthase (NOS2).

Compared to the murine system, much less is known about the activation of anti-mycobacterial activity in human macrophages. Individuals functionally deficient in  $IFN-\gamma$  as a result of mutation in the  $IFN-\gamma$  receptor gene are more susceptible to mycobacterial infections, supporting a significant role for this cytokine in defense against MTB in humans. *In vitro* evidence supporting a role for  $IFN-\gamma$  in defense against human TB has been scarce due to the lack of an experimental system in which the killing of MTB by macrophages can be reproducibly demonstrated. Thus, reports of the effect of  $IFN-\gamma$  treated human macrophages on the replication of MTB range from its being inhibitory [114] to enhancing [38]. This inconsistency cast



considerable doubt on the anti-mycobacterial capability of human mononuclear phagocytes until the demonstration that 1,25-dihydroxy vitamin D<sub>3</sub> [1,25-(OH)<sub>2</sub>D<sub>3</sub>], alone or in combination with IFN- $\gamma$  and TNF- $\alpha$ , was able to activate macrophages to inhibit and/or kill MTB in the human system [34]. 1,25-(OH)<sub>2</sub>D<sub>3</sub> is known to have an immuno-regulatory role mediated through binding to the vitamin D receptor in immune cells including macrophages [111]. Not surprisingly, the production of RNI by human macrophages has also been difficult to demonstrate, although there are reports of NOS2 induction and/or RNI production by human macrophages from various groups [47]. Recently, 1,25-(OH)<sub>2</sub>D<sub>3</sub> was reported to induce the expression of the NOS2 and MTB inhibitory activity in the human HL-60 macrophage-like cell line [113]. This observation thus identifies NO and related RNI as the putative anti-mycobacterial effectors produced by human macrophages. This controversial notion is further supported by recent evidence that IFN- $\gamma$  -stimulated human macrophages co-cultured with lymphocytes exhibit mycobacteriocidal activity concomitant with the expression of NOS2. Moreover, it was also reported that human monocytes are unable to generate tetrahydrobiopterin, which is an essential cofactor for arginine-dependent NO synthesis [135]. Finally, it is worth to note that all these studies have been carried out with blood monocytes derived macrophages (MDM), not tissue macrophages. To this regards, other human cell type, e.g., endothelial cells, liver cells can produce this NO in large quantities. Also it is not clear that whether human macrophages are unable to do so or whether the correct combination of inducers and culture conditions or tissue sources has not yet been utilized.

Another anti-mycobacterial mechanism of macrophages is phagolysosome fusion. Lysosomes, in the late endocytic pathway, contain numerous hydrolytic enzymes and are a very acidic organelles. Fusion of the lysosome with phagosome-containing ingested bacteria is a primary mechanism by which macrophages control infections. Phagolysosome fusion is increased when the macrophage is activated with IFN- $\gamma$  or other cytokines. MTB is initially within a phagosome, and has been shown to inhibit phagolysosome fusion and acidification in "non-activated" macrophages, and thereby avoid being killed by the macrophage. [47].

## 5. Binding and uptake of *M. tuberculosis*

MTB enters the human host via the respiratory route, where it encounters the alveolar macrophages. These cells are both the host and the first line of defenses against the pathogen. Macrophages, as professional phagocytes, are endowed with several surface receptors that facilitate antigen uptake, thereby rendering specific host-invasion strategies dispensable. Cholesterol has been shown to act as a docking site for the pathogen, promoting receptor-ligand interactions. The initial interaction with surface receptors influences the subsequent fate of MTB within the macrophages. Interaction with the constant regions of immunoglobulin receptors (FcRs) and Toll-like receptor (TLRs) stimulate host-defense mechanisms such as oxidative burst and RNI production, where as those with the complement receptors (CRs) or macrophage mannose receptors (MMRs) promote the mycobacterial survival [72]. MTB however, preferably enters the macrophages through CRs (CR1, CR3, and CR4), and the MMRs assisted phagocytosis. Recent studies have shown that the CR3 (CD11b/CD18) and CR4 (CD11c/CD18) and MMRs are also capable of traducing intracellular signals [42], suggesting that binding of MTB to these receptors may lead to cellular activation, thereby bacterial death. MTB on the other hand solves this obstacle by arresting the maturation of phagosome at the early stage, and by preventing fusion of phogosome with the lysosome [43]. Mycobacteria may also attach to macrophages through fibronectin-vitronetin interaction, by secreting some fibronectin binding proteins [1].

Finally, one common hypothesis proposes that MTB evades killing by entering macrophage through receptors that are unable to activate cellular microbiocidal activity.

## 6. Intracellular fate of *M. tuberculosis*

Following attachment and subsequent phagocytosis of MTB, its intracellular survival and sustained growth initially depends on bacillus ability to avoid destruction by free radicals (i.e., ROIs, RNIs), lysosomal enzymes, and other cytokines mediated responses. Mycobacterial cell components that nullify the toxic effect of free radicals or the mechanisms that block the fusion



of mycobacteria containing phagosomes with lysosomes could be critical for this survival. The production of free radicals and its role in macrophage innate immunity as well as the dynamics of phagosome-lysosome fusion and its effects on intracellular bacterial replication in infected human macrophages have been studied by various groups, which in brief could be described below.

## 6.1 Free radical generation and mycobacterial survival mechanisms

High output nitric oxide (NO) and ROIs production by immunologically activated macrophages are shown as major antimicrobial mechanisms [46]. However, ROIs are not likely to be involved in macrophage mycobactericidal activities, although the production of RNIs by murine macrophages is demonstrated to be an important antimycobacterial effector mechanism. On the other hand, in response to these aggressive antimicrobial molecules, bacteria have evolved a multitude of resistance mechanisms to promote a dynamic balance during infection. Moreover, if the microorganisms do not manage to evade physical contact with these highly reactive molecules, their survival depends on detoxification mechanisms, including production of molecular scavengers, antioxidant enzymes, repair systems, and expression of specific antioxidant regulons.

**a) Reactive nitrogen intermediates:** mononuclear phagocytes, upon activation by appropriate cytokines such as IFN- $\gamma$  and TNF- $\alpha$ , generate NO and related RNI via iNOS2 using L-arginine as the substrate. The significance of these toxic nitrogen oxides in host defense against MTB has been well documented, both *in vitro* and *in vivo*, particularly in the murine system [46]. However, despite the ability of human macrophages to produce RNI in quantities sufficient to effect killing of *M. avium* *in vitro*, efforts to demonstrate the activation of the L-arginine-dependant cytotoxic mechanism in these human's cells have, in general, generated inconsistent results. Could this be due to the known insufficiency of tetrahydrobiopterin in human macrophages [151]? Which reversed the negative effect of catalase on RNI production? More importantly, accumulating evidence strongly supports a role for RNI in host defense in human TB [148], as high-level expression of iNOS2 has been detected immunohistochemically in

macrophages obtained by pulmonary alveolar lavage from individuals with active pulmonary TB [148]. Interestingly, although IFN- $\gamma$  and TNF- $\alpha$  is sufficient to activate the L-arginine-dependent pathway in murine macrophages, optimal RNI production by human hepatocytes requires IL-1 and lipopolysacchrides (LPS) in addition [102]. This variation in inducing agent's requirement, for RNI production, by cells of different origins highlights the possibility that human macrophages iNOS2 may require as yet undefined correct combination of inducers and culture conditions or tissue sources. Finally, it is worth to note that most of these studies have been carried out with blood monocytes, not tissue macrophages. Since other human cell type, e.g., endothelial cells, liver cells can produce this NO in large quantities; further studies are required incorporating different macrophages sources.

From mycobacterium's point of view, the development of strategies to evade the toxic effect of RNI seems prudent, if not absolutely required, for survival. Mannocylation of LAM ('i.e.' Man-LAM) in virulent strain avoids the production of TNF- $\alpha$  by macrophages which is a key cytokines for the induction of RNI response. Balanced iron levels inside the phagocyte are of particular relevance to both the pathogen and the host cell. Although, iron is an essential nutrient for optimum growth of bacteria, it also functions as an important cofactor of microbicidal effector mechanisms. In this context, MTB utilizes the host iron for its own growth by producing salicylic acid derived soluble iron-chelators called mycobactins, resulting in arrest of Fe dependent L-arginine-NO anti-mycobacterial pathway.

**b) Reactive oxygen intermediates:** while the role of macrophage iNOS2 in host defense against MTB is well established, the significance of toxic oxygen species in the control of TB remains controversial. Despite the demonstration that H<sub>2</sub>O<sub>2</sub> generated by cytokine-activated macrophages was mycobacteriocidal [146], the ability of ROI to kill MTB remains to be confirmed [44]. Indeed, mycobacteria are capable of evading the toxic effect of ROI by various means [46]. For example, mycobacterial components lipoarabinomannan (LAM) and phenolicglycolipid I (PGL-I) are potent oxygen radical scavengers [19, 46]. In this regard, LAM a major cell wall associated phosphatidylinositol-anchored complex LPS, which is produced

by MTB in large amounts (15mg/g of bacteria), has been shown to incapacitate the oxygen radical dependent antimicrobial mechanisms at at-least three ways: i) Electron spin resonance spectroscopy and spin-trapping studies have shown that LAM is an effective oxygen scavenger, ii) LAM can down-regulate the oxidative burst by inhibiting protein kinase C, [19], and iii) LAM virtue of its ability inhibits transcriptional activation of IFN- $\gamma$  inducible gene [19]. Other mycobacterial components that interfere with the oxygen radical dependent anti-microbial mechanism of macrophages are PGL-I and the sulfatides. Although, universally distributed among the *M. leprae*, the expression of PGL-I in various strains of MTB is much restricted [12]. Because of its restricted distribution among tuberculous isolates, the significance of PGL-I in the pathogenesis of TB remains to be determined. In contrast, the sulfatides, derivatives of multiacylated trehalose 2-sulfate, are widely expressed among different strains of MTB [58]. Nonetheless, both PGL-I and the sulfatides have the capacity to down-regulate ROI production in *in-vitro* macrophage culture systems, and PGL-I has shown to directly scavenge oxygen radicals in a cell free system. Another mechanism by which MTB could evade the toxicity of ROI is to avoid binding to macrophage cell surface components, such as Fc receptors, that would otherwise provoke an oxidative burst.

## **6.2 Phagolysosome fusion and mycobacterial escape strategies**

Fusion of lysosomes with phagosomes containing ingested bacteria is a primary mechanism by which macrophages control infections. The lysosome is a complex vacuolar organelle of the late endocytic pathway. Within the lysosomal vacuoles there are potent hydrolytic enzymes capable of degrading a whole range of macromolecules including microbes. These enzymes function optimally at acidic pH, a condition found within the intralysosomal milieu. The lysosome is, in fact, the most acidic organelle in animal cells with pH 4.5-5.0 and this acidic environment is maintained by membrane ATP-dependent proton pumps, the vacuolar H<sup>+</sup>-ATPases [94].

Formation of phagolysosomes is, in general, a dynamic process during which the phagosome matures, modified by transient fission and fusion with endocytic organelles before to fuse with lysosomes [36]. Phagocytosed microorganisms are subject to degradation by intralysosomal acidic hydrolases upon phagolysosome fusion. It appears that the antimicrobial activity of the phagolysosome is mediated, at least in part, by the degradative function of lysosomal hydrolases and/or direct and indirect effect of acidification. However, the precise mechanisms by which the hydrolases and acidification confer antimicrobial properties, as well as the process of acidification of the various endocytic compartments, are not completely understood [94]. Significant progress has been made in this area of mycobacterial research since D'Arcy Hart and colleagues hypothesized that prevention of phagolysosomal fusion is a mechanism by which MTB survives inside macrophages [5]. In this context, early studies focused on MTB products that might disrupt phagolysosome fusion and has been reported that mycobacterial sulfatides [57], derivatives of multiacylated trehalose 2-sulfate, a lysosomotropic polyanionic glycolipid [58], have the ability to inhibit phagolysosomal fusion. *In vitro* studies demonstrated that MTB generates copious amounts of ammonia in cultures (concentrations of up to 20 mM have been detected) and is thought to be responsible for the inhibitory effect of culture supernatants of virulent mycobacteria on phagolysosome fusion [56]. This finding is in keeping with the observation that the weak base ammonium chloride affects salutatory movement of lysosomes as well as alkalinizes the intralysosomal compartment [56]. However, it is unlikely that the ability of ammonia to alkalinize intracellular vacuoles accounts for its inhibitory effect on phagolysosome fusion because other bases capable of raising intralysosomal pH actually promote phagolysosome fusion [56]. Thus, the precise mechanisms by which ammonia prevents phagolysosome fusion remain to be determined. Nevertheless, by virtue of its ability to produce significant amounts of ammonia, the tubercle bacillus can evade the toxic environment within the lysosomal vacuole by inhibiting phagolysosome fusion and diminishing the potency of the intralysosomal enzymes via alkalization.

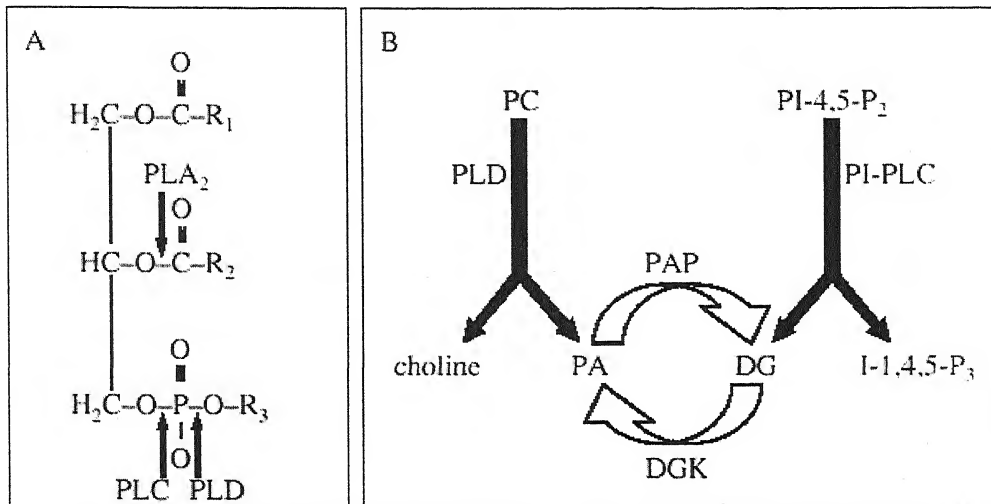
Vacuolar ATPase, normally present in maturing phagosomes, is thought to be acquired from endosomal compartments [94]. The exclusion of vacuolar ATPase proton pumps from phagosomes containing live MTB or *M. avium* [136] provides a mechanism for the relative lack of acidification of mycobacterial phagosomes. While it remains to be proven whether alkalization of mycobacterial vacuoles attenuates phagolysosomal fusion, increase in intralysosomal pH is likely to help mycobacteria evade the adverse effect of an otherwise acidic environment.

Recent work on the effect of the tubercle bacillus on the fusigenicity of phagosomes and lysosomes has focused on the mechanisms underlying the alteration of biochemical properties of vacuolar membrane components and intravacuolar contents. For example, the GTPases of the Ras family, known to play a role in the interaction between various endocytic compartments [36], have been a target of investigation aimed at understanding the inability of mycobacterial phagosomes to mature to phagolysosomes. Thus, mycobacterial phagosomes retain Rab5, which plays a role in the interaction between early endocytic compartments and phagosomes [36], and exclude Rab7, a GTPase that regulates late endosomal membrane trafficking [37]. Moreover, compared with the other phagocytic particle studied, the MTB phagosomes exhibited delayed clearance of major histocompatibility complex (MHC) class I molecules, relatively high levels of MHC class II molecules. Furthermore, studies showed that phagosomes containing MTB retains higher levels of markers for early endosomes such as transferring receptor (TFR), and relatively low levels -of late endocytic markers such as lysosomal membrane glycoproteins (CD63, LAMP-1, and LAMP-2) and -of the lysosomal acid protease cathepsin D. In contrast, phagosomes containing either polystyrene beads or heat-killed MTB stained intensively for LAMP and cathepsin D. These findings suggest that viable MTB actively blocks the maturation of its phagosomes along the endosomal lysosomal pathway. All together these data also demonstrate that MTB arrests the maturation of its phagosomes as a stage at which the phagosome interacts with early and late endosomes but not with lysosomes [22].

The recent discovery of the TACO (*t*ryptophan *a*spartate-containing *co* at) protein provides the most succinct and direct mechanistic explanation for the inability of mycobacteria-containing phagosomes to fuse with lysosomes [43]. Analysis of the biochemical composition of mycobacterial phagosomes obtained by organelle electrophoresis of a microsomal fraction prepared from S-methionine/cysteine-labeled, BCG-infected macrophages has identified a 50-kDa host cell polypeptide specific for phagosomes containing live bacilli. This polypeptide was not detected in other subcellular fractions analyzed, including the phagosomes that contain dead BCG, nor was it present in metabolically labeled bacteria. TACO contains 5 WD [Trp-Asp] repeats and exhibits homology to coronin, a *Dictyostelium discoidium* WD repeat actin-binding protein involved in actin-based cytoskeletal rearrangements. TACO is present in lymphoid and myeloid cells, and it was associated with the cortical microtubule network in non-infected macrophages. By 2 hours post-infection with BCG, TACO was almost completely re-localized (from the cortical distribution) to the BCG-containing phagosomal membrane and it remained associated for a prolonged period of time. TACO was also detected in phagosomes containing dead BCG. However, the association was transient and dissociation occurred within 2 hours after phagocytosis. Thus, viability of phagocytosed bacilli is requisite to the retention of TACO. This process is not due to the lack of acidification of phagosomes containing live BCG because those containing dead bacilli, in the presence of  $\text{NH}_4\text{Cl}$ , a phagosome-alkalinizing agent, failed to retain TACO. By retaining TACO and thus intercepting the fusion of phagosome with lysosome, mycobacteria evade potent lysosomal antimicrobial functions of macrophages. Intriguingly, TACO is not expressed in Kupffer cells, the resident phagocytes of the liver, and may well account for the relative resistance of this organ to MTB infection. Elucidation of the mechanisms by which MTB contains phagosomes and retains TACO will provide insight into the pathogenesis of the tubercle bacillus.

## 7. Phospholipase

Phospholipases are enzymes that hydrolyze ester bonds of various phospholipids to generate different byproducts as a second messenger. The mammalian phospholipases are phospholipase A<sub>2</sub> (PLA<sub>2</sub>), phospholipase C (PLC) and Phospholipase D (PLD), which are names after they cleave, as shown in **figure 5**.



**Figure 5. A: Bonds hydrolysed by phospholipases A<sub>2</sub> (PLA<sub>2</sub>), C (PLC) and D (PLD).** R<sub>1</sub> and R<sub>2</sub> represent esterified fatty acids, e.g. oleic, palmitic, or arachidonic acid. R<sub>3</sub> represents the polar head of the phospholipid, e.g. choline or inositol 4,5-bisphosphate. **B:** Interconversion of phosphatidic acid (PA), and diacylglycerol (DG), generated by PLD and phosphatidylinositol-specific PLC (PI-PLC). DGK: diacylglycerol kinase; PAP: phosphatidic acid phosphohydrolase; PC: phosphatidylcholine; PI-4,5-P<sub>2</sub>: phosphatidylinositol 4,5-bisphosphate; I-1,4,5-P<sub>3</sub>: inositol-1,4,5-trisphosphate.

In eukaryotic cells, these enzymes can evoke both unique and common signals. Metabolism of phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) by the phosphatidylinositol (PI)-specific PLC (PI-PLC) enzymes produces the second messengers, inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). These two second messengers are known to modulate intracellular events through their respective regulation of intracellular free Ca<sup>++</sup> and protein kinase C (PKC) isozymes. Hydrolysis of phospholipids by PLD produces phosphatidic acid (PA) and the respective head groups. PA, besides acting as a precursor for DAG, has been proposed to modulate a variety of intracellular events including actin polymerization, mitogenesis, and protein trafficking [126]. The primary glycerol based product of PLC and PLD, DAG and PA respectively, can be interconvert by DAG kinase and PA phosphohydrolase. Conversion of PA

to DAG is a mechanism for returning the molecule to the phospholipid pool via the Kennedy pathway, and phosphorylation of DAG derived from  $\text{PIP}_2$  forms PA for re-synthesis of PI. The sub-cellular localization of these activities and their coordinated regulation are anticipated to be vital for the modulatory events allowed in individual cells.

## 7.1 Phospholipase D

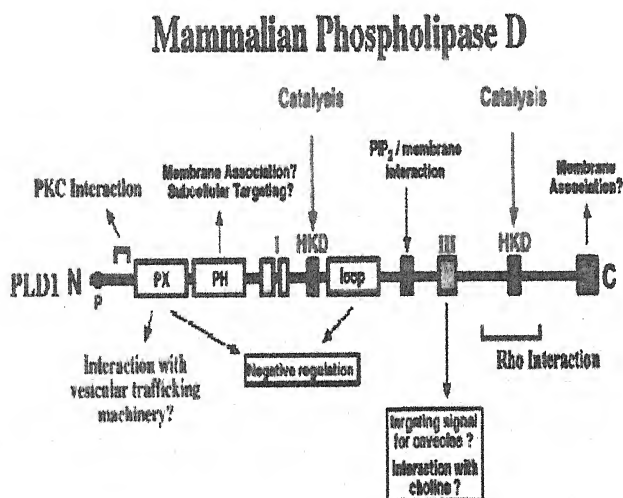
PLD is a hydrolytic enzyme that catalyses the hydrolysis of the major membrane phospholipid (i.e. Phosphatidylcholine; PC) by cutting the ester bonds, to generate PA and choline. PLD activity was initially observed in cabbage leaves in 1948, and has proven to be wide spread throughout both prokaryotes and eukaryotes. Nearly thirty years later, it was detected in mammalian tissues, first in the rat brain [117]. Thereafter, PLD has been demonstrated in virtually all mammalian cells and tissues studied. Bovine adrenal chromaffin cells have been claimed to be an exception, and whether they possess PLD activity is under debate [16].

The first mammalian PLD was cloned and characterized from HeLa cell cDNA library and found to encode a 1074-aminoacid protein. The expressed  $\text{PLD}_1$ , exhibits PC-specific PLD activity (hydrolysis and transphosphatidylation) and was shown to be activated by ADP-ribosylation factor (ARF) and by  $\text{PIP}_2$ .  $\text{PLD}_1$  has been renamed  $\text{PLD}_{1a}$  after another splice variant has been identified,  $\text{PLD}_{1b}$ , that lacks a 38-aminoacid region [59]. These two isoforms share some biochemical properties, such as positive regulation by  $\text{PIP}_2$  and inhibition by oleate [40]. Nevertheless, the two isoforms are differentially regulated by PKC, and the small GTPases ARF and Rho [40]. A distinct PLD isoenzyme,  $\text{PLD}_2$ , has also been cloned. It is constitutively active and appears to be regulated mainly by inhibition [73]. Furthermore,  $\text{PLD}_1$  and  $\text{PLD}_2$  are only 50% identical, localize to different intracellular compartments and display differences in terms of both basal activity and sensitivity to intracellular activators and inhibitors [40]. There is also evidence suggesting that at least one more type, dependent on oleic acid but independent of phosphoinositides, remains to be cloned [40, 73].



### 7.1.1 Structure and enzymology

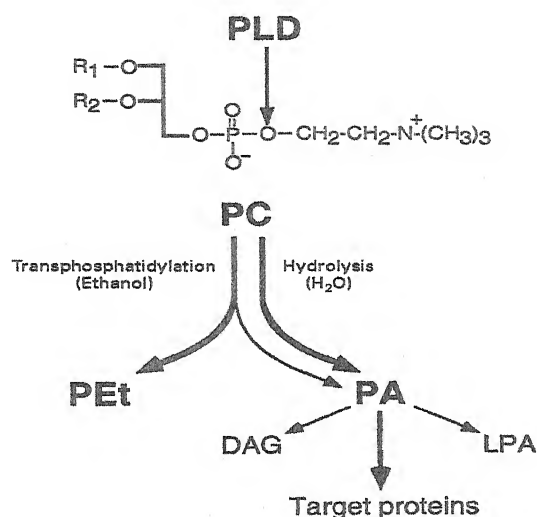
PLDs and its sequences from representative of diverse species (such as vertebrate, invertebrate, fungal, plant, and bacterial species) display notable similarities -to each other, -to PS, -to cardiolipin synthases, -to endonucleases, and -to Poxviridae proteins of unknown function [83]. All eukaryotic PLD genes cloned to date share a relatively conserved catalytic core flanked with much less conserved N- and C-terminal regions. The catalytic core of all eukaryotic PLD is comprised of domains I-IV, as shown in the **figure 6**. These domains are found also in bacterial PLD. There is significant internal similarity, in four short sequence motifs, between domains I plus II and domains III plus IV. This suggests that eukaryotic PLD genes evolved from an ancestral gene that underwent a gene duplication and fusion event, raising the possibility that PLDs are bilobed enzymes. In addition to domains I-IV, at N terminal an additional domains called PX (*phox*) domain, with yet fully unknown function, are conserved in the yeast, human and nematode sequences, but are absent from the plant and the bacterial PLDs. Analysis of the amino acid sequence of PLD<sub>1</sub> reveal that domain IV contains a relatively short sequence motif HXXKX4DX5IGSXN, termed the HKD motif [59] or the phosphatidyltransferase motif [145], believe to be important for catalytic activity of enzyme.



**Figure 6. Schematic depiction of human PLD<sub>1</sub>.** Boxes, location of regions of highly conserved sequences or regions unique to PLD<sub>1</sub> (loop sequence). Courtesy: [49].

The HKD motif is particularly well conserved in eukaryotic and bacterial PLDs, but can be found also in other PLD superfamily members such as non-PLD phosphatidyltransferase (e.g. cardiolipin synthase and phosphatidyl serine synthase), as well as in certain pox virus envelope proteins and several endonuclease [98]. Because of their high degree of conservation, it has been predicted that the histidine, lysine, aspartate, glycine, and serine residues in HKD motifs play an important role in catalysis, which has been largely confirmed from the mutagenesis studies. Recent studies, in an endonuclease superfamily member and in the *Yersinia pestis* murine toxin (which has PLD activity), have identified the histidine in the HKD motif as a nucleophile that forms a covalent phosphor-histidine intermediate [116]. This result suggest that, in PLDs, this histidine residues attaches the phosphatidyl moiety to form a transient phosphatidyl enzyme

intermediate, which is then subject to attack by an activated water molecules to release PA or by a primary short- chain alcohol to release a phosphatidyl alcohol [116].



**Figure 7. Transphosphorylation reaction.** PLD in presence of ethanol converts the PC into Pet-OH, instead of PA.

In fact, a unique property of PLD is the preference for primary alcohols rather than water in the catalysis reaction. Thus, PLD activity can lead to the generation of phosphatidylethanol (Pet-OH) rather than PA in the presence of 1-2% ethanol. This reaction, referred to as transphosphatidylation, is not only a hallmark of PLD activity but has also been used to block the production of PA in functional studies (Figure 7).

### 7.1.2 Regulation of PLD activity

Although PLD activity is distributed widely in animals, plants, fungi and bacteria, study of its regulation have been mainly confined to mammalian tissues and cells. Its activity in mammalian cells is low and is transiently stimulated by various hormones, neurotransmitters, growth factors, cytokines, and other agonists that activate various cell surface receptors. The diverse nature of stimulatory agonists suggest that the enzyme is regulated by various signaling mechanisms including those of -Rho family small G proteins heterotrimeric G proteins, -tyrosine kinase family, -PK family especially C, -ARF, and -calcium ions.

In some system, PLD activity can be increased by guanine nucleotides with the pharmacologically selectivity expected for G protein-mediated process. Recent works identified members of the ARF family of G proteins as guanine-nucleotide dependent PLD activators in HL-60 cells and human neutrophils membranes [98]. ARF proteins belong to the Ras superfamily of monomeric GTP-binding proteins. ARF was originally identified as a protein cofactor required for efficient ADP-ribosylation of the  $\alpha$ -subunit of  $G_s$  (the heterotrimeric G protein that stimulates adenylyl cyclase) by cholera toxin. This activity is characteristic of all six identified mammalian ARF proteins. More recently, the ARF proteins have been shown to have an essential role in vesicular membrane trafficking in eukaryotic cells.

Another monomeric G protein, Rho A (acting in concert with an unidentified cytosolic factor), was demonstrated to mediate PLD activation in human neutrophils membranes. The Rho family of monomeric G proteins comprises a second class of cytosolic GTP-dependent activators of PLD. Members of the Rho family (RhoA, B, C, and G; Rac1 and 2; CDC42; and TC10) play various roles in cellular processes involving cytoskeletal rearrangements, membrane movement, and cell growth. Moreover, subsequent studies have revealed Rho family of G proteins, act synergistically with ARF to activate PLD, suggesting the existence of a single enzyme that is responsive to both G proteins activators [126]. At present it is unclear how (or if) these G proteins participate in the mechanism by which cell-surface receptors activate PLD

enzymes. However other worker implicates lipid cofactors and proteins kinases as intermediates in the activation cascade.

Thereafter, it has been demonstrated that the ARF and Rho-responsive PLD activities are potently and specifically stimulated when PIP<sub>2</sub> is included in the phospholipids vesicles containing radiolabelled PC as a substrate. A requirement of PIP<sub>2</sub> for PLD activity *in vivo* was suggested in experiments with permeabilized U937 cells [126].

PKC is clearly a major regulator of PLD in intact cells, and phorbol esters which mimic DAG and activate PKC, have been shown to activate PLD in numerous cells [40]. Over-expression or down-regulation of PKC in various cells has led to an increase or attenuation of hormone-regulated PLD activity, respectively, and thus suggests a functional role for these enzymes. However, the precise role of these proteins is unclear, especially in view of the variable efficacy of PKC inhibitors in reversing the effects of PMA in various systems [126]. Activation of PLD by PKC in membranes from HL-60 cells also required ATP but was not affected by a catalytic site inhibitor of PKC activity. A subsequent study suggested that the actual role of ATP in this system is to maintain elevated levels of PIP<sub>2</sub> in the membranes, presumably to support PLD activity as a cofactor [126].

Tyrosine kinase activity represents an alternative mode of regulation for PLD. Numerous studies indicate a role of protein tyrosine phosphorylation in growth factor-, agonist-, and oxidant-mediated activation of PLD. In the case of growth factors, stimulation of PLD often follows activation of PLC and PKC in response to receptor tyrosine phosphorylation. Activation involving tyrosine phosphorylation also occurs by mechanisms that are independent of PKC [40, 126].

Some structural and regulatory properties of cloned PLDs and oleate-dependent PLD activity are summarised in **table 1**.

	<b>PLD1a, PLD1b</b>	<b>PLD2</b>	<b>Oleate-dependent PLD</b>
Size	124 kDa (a), 119 kDa (b)	106 kDa	190 kDa <sup>a</sup>
Preferred membranes	Golgi membranes, microsomes, endosomes	plasma membrane (PM)	PM, nucleus, microsomes
Cytosolic	probably	possibly	no
Basal activity	low	high	low
Activation by			
*lipids	PIP2, PIP3	PIP2, PIP3	oleate, arachidonate
*GTPases	Arf, RhoA, Rac, Cdc42	minor	no
*kinases	cPKC $\alpha$ , $\beta$ , nPKC $\epsilon$	no	no
Inhibitory proteins	synaptojanin, fodrin, AP3	synaptojanin, synuclein	not known
Other inhibitors	oleate, primary alcohols, ceramides	oleate, primary alcohols	primary alcohols

**Table 1 . Characteristics of phospholipase D enzymes and activities.**

References: [40, 59, 73, 83, 98, 126].

<sup>a</sup>size approximated from electrophoresis gels.

cArf reportedly activates a truncated PLD2

Although  $\text{Ca}^{++}$  ions can stimulate the activity of certain PLD isoforms, the concentrations required are often well above the physiological cytosolic range or the stimulation is not observed in the presence of physiological  $\text{Mg}^{++}$  concentrations. Thus, direct control of the enzyme by physiological changes in cytosolic  $\text{Ca}^{++}$  seems unlikely. However, many studies have shown that depletion of cellular  $\text{Ca}^{++}$  by treatment of cells with  $\text{Ca}^{++}$  chelators (EGTA or BAPTA) results in inhibition of the activation of PLD by various agonists, and that  $\text{Ca}^{++}$  ionophores can activate PLD in many cell types [40]. Furthermore, detailed studies of the intracellular concentration of  $\text{Ca}^{++}$  required for activation of PLD in permeabilized or intact cells have indicated that increases in the physiological range (from 0.1 to 1  $\mu\text{M}$ ) are effective [40]. The  $\text{Ca}^{++}$ -dependent protein that regulates PLD has not been defined, but there is evidence that calmodulin can regulate the enzyme. Other possibilities are the  $\text{Ca}^{++}$ -dependent PKC isozymes and tyrosine kinases.

### 7.1.3 Cellular functions of PLD

Rapid and transient increases in PA, attributed to agonist-stimulated PLD activity, have been observed in a variety of cell lines. PA can be metabolized further to bioactive long lasting lipids like DAG (through the action of PA phosphatase) [40] or to potent mitogenic agonist lysophosphatidic acid (through the action of a selective PA-hydrolyzing PLA<sub>2</sub>) [126]. Therefore, elucidation of roles for PLD in cellular regulation has focused on the identification of cellular targets affected by its reaction product, 'i.e.' PA. Important regulatory proteins whose activities can be activated directly by PA in an *in vitro* system include protein kinases, protein tyrosine phosphatase, PLC, phosphoinositol-4-kinase, Raf-1 kinase and small molecular weight guanosine triphosphate-activating proteins [126]. Whether any of these enzymes are regulated by PA *in vivo* remains to be determined. If regulation of target enzymes by increased intercellular PA is established, it will be important to determine whether the lipid is produced by PLD, the action of DAG kinase, or de novo synthesis via fatty acylation of glycerol-3-phosphate. However, accumulated evidences suggest that activation of PLD is probably the major pathway for signal-evoked PA formation. PA can also act as second messenger, because a reported direct role -in secretory vesicle budding [125] and -in activation of phagocyte responses, including degranulation [160], phagocytosis [78], respiratory burst [40]. Moreover, the expression and activation of PLD, thus PA generation, has been recently reported to be actively associated with the phagocytosis [78] and killing [77] of virulent strains of mycobacteria by human macrophages.

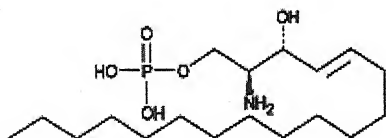
## 8. Sphingolipids

Sphingolipids are complex lipids found in all mammalian cells and are mostly located in the plasma membrane. They all contain a long-chain base -the sphingoid base (mostly sphingosine) as a backbone, -an amide linked fatty acid of varying chain length (usually, long or very long chain), and -one of various polar head groups (such as hydroxyl for ceramide, phosphorylcholine for sphingomyelin, and carbohydrate residues for glycosphingolipids). Long chain bases

(sphingoids or sphingoid bases) are the characteristics or are the defining unit of the sphingolipids.

The root term “sphingo” was first coined by J.L.W. Thudichum in 1884 because the enigmatic nature of the molecules reminded him of the riddle of the sphinx. Since their discovery over 100 years ago, sphingolipids have caught the eyes and the imagination of scientists. Modern science has made many new insights on the cell biology and day-to-day functions of many integral sphingolipids, especially those of small lipid messengers. Several sphingolipids and its metabolites are recognized as a vital second messenger in the signal transduction process mediated by receptors of many cytokines and growth factors. A great part of current understanding of these lipid metabolites has been achieved from apoptosis-related studies. However, recent data in the fields of immunology, endocrinology and neurobiology, also suggest a fundamental involvement of lipid metabolites in the onset of diseases. Although sphingolipids were originally thought to play a predominantly structural role as components of the lipid bilayer, sphingolipid metabolism is now known to be a dynamic process and sphingolipid metabolites-including ceramide, sphingosine, and sphingosine 1-phosphate (S1P) are active mediators that play essential roles in cell growth, survival, differentiation, proliferation, death and various cellular functions (such as  $\text{Ca}^{++}$  homeostasis).

### 8.1 Sphingosine 1-phosphate

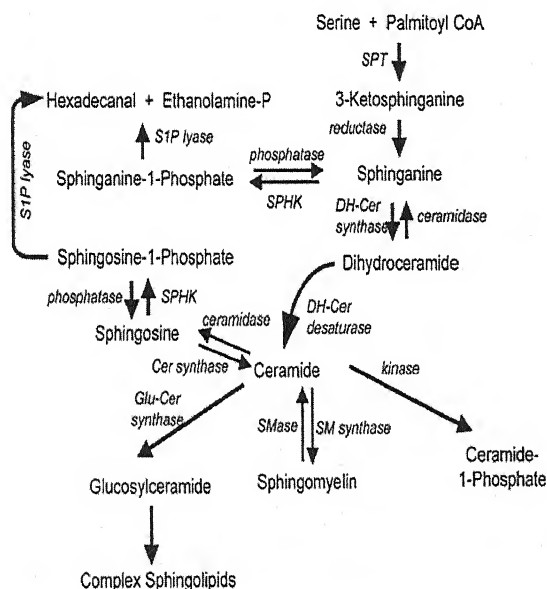


Sphingosine 1-phosphate (S1P), a product of sphingolipid metabolism, known to regulate growth/survival, differentiation, chemotaxis of various cell types, has been suggested to be a unique signaling molecule due to its both intracellular and extracellular mode of action. Although its intracellular targets have not yet been unequivocally identified [131], evidences have been reported that S1P may function as a second messenger important for regulation of  $\text{Ca}^{++}$  homeostasis [95] and suppression of apoptosis [28]. Extracellularly, S1P is prevalently associated with serum high density lipoprotein (HDL) [99] and can provide signal to the target cells through endothelial differentiation gene (EDG) receptors [107]. After binding

with cognate receptors, S1P is responsible for much of the lipid-derived biological activity of serum, such as proliferation, survival, stress fiber and focal adhesion plaque formation, fibronectin matrix assembly, cell rounding and cell migration [130]. Moreover, S1P has been reported to trigger multiple signaling pathways, including activation of phospholipases, such as PLD [35], whose role in antimicrobial activity has been reported [77]. Finally, the induction of respiratory burst by S1P in alveolar macrophages has also been shown, suggesting that S1P could also play a role as physiological activator of alveolar macrophages [63].

S1P is stored and released from platelets upon their activation, but can also be synthesized in a wide variety of cell types in response to extracellular stimuli, such as growth factors and cytokines, G-protein-coupled receptors (GPCR) agonists and antigens.

### 8.1.1 Biosynthesis and metabolism of S1P



**Figure 8. Sphingolipid metabolism.** Cer, ceramide; DH-Cer, dihydroceramide; DH-SPH, dihydro-sphingosine or sphinganine; Ethanolamine-P, phosphorethanol-amine; SMase, sphingomyelinase; SM, sphingomyelin; S1P, sphingosine-1-phosphate; SPHK, sphingosine kinase; SPT, serine palmitoyltransferase

S1P can be synthesized either in response to the agonist-dependent activation of sphingomyelinases or *de novo*. Agonist-dependent activation of sphingomyelinase has been observed in response to growth factors, cytokines and arachidonic acids [82]. Additionally, cellular stress and changes in the redox state of the cell can result in sphingomyelinase activation. Upon cellular activation sphingomyelinases converts the sphingomyelin to ceramide. Ceramidase catalyzes the deacylation of ceramide to produce non estrified fatty acid and



sphingosine. The resulting sphingosine may act as a substrate for sphingosine kinase (SPHK) to produce S1P. This is then cleaved by S1P lyase to produce palmitaldehyde and phosphoethanolamine or dephosphorylated by S1P phosphatase to produce sphingosine. Alternatively, sphingosine can be converted back into ceramide and then ceramide into sphingomyelin by the addition of a phosphocholin head group (donated by phosphatidylcholine), catalysed by ceramide synthase and sphingomyelin synthase respectively. These interchangeable molecular relationships are illustrated as sphingomyelin cycle in the **figure 8**.

*De novo* S1P synthesis begins with the biosynthesis of sphinganine (which occurs in the endoplasmic reticulum), that involves the condensation of the serine and palmitoyl-coenzyme A to produce 3-oxo-sphinganine and CO<sub>2</sub>, catalyzed by serine palmitoyltransferase. The keto group of 3-oxosphinganine is rapidly reduced to hydroxyl dihydrosphingosine (sphinganine) by an NAD(P)H- dependent reductase, a step that must occur rapidly as intermediates are rarely encountered. Subsequently, free dihydrosphingosine (sphinganine) is rapidly N-acylated to form a dihydroceramide, by a dihydroceramide synthase, which can utilize a range of acyl-CoAs. Dihydroceramide desaturase catalyses the subsequent introduction of a trans double bond at C-4-C-5 to produce ceramide. Further ceramide can be converted to sphingosine and S1P as described above.

### **8.1.2 Extracellular actions of S1P**

S1P known to play important roles in the regulation of a variety of biological processes, including Ca<sup>++</sup> mobilization, reorganization of the cytoskeleton, as well as cell growth, differentiation, survival and motility. Similar to other important phospholipids mediators, S1P exerts dual actions in cells: it acts intracellularly as a second messenger and extracellularly as a ligand for specific GPCRs. Thus, S1P is the natural ligand for the specific GPCRs that were originally known as the EDG-1 family of proteins but were recently renamed S1P receptors (S1PRs) [107]. To date, five members of the S1PR family have been cloned –including S1P<sub>1</sub> (EDG-1), S1P<sub>2</sub> (EDG-5), S1P<sub>3</sub> (EDG-3), S1P<sub>4</sub> (EDG-6), and S1P<sub>5</sub> (EDG-8). All of these receptors bind and are activated specifically by S1P and by dihydroS1P (also known as

sphinganine 1-phosphate dihydro, whose structure is identical to that of S1P with the exception that it lacks the 4,5-trans-double bond). Members of this family of the receptors are differentially expressed on different tissues, and couple to a variety of G proteins. As a result, S1P can potentially activate and regulate a diverse array of signal transduction pathways in different cells types as well as within the same cells, resulting in the possibilities of the diverse biological outcomes, depending on the relative abundance of S1PRs and associated G proteins and cell type.

One of the more widely studied functions of extracellular S1P is the regulation of cell migration and its role in angiogenesis. S1P stimulates directed migration of endothelial cell and vascular smooth muscle cells, critical events in the formation and extension of new blood vessels, as well as promoting capillary-like tube formation by bovine aortic endothelial cells [105]. These events appear to be mediated primarily by binding of S1P to S1P<sub>1</sub> and the subsequent activation of a pertussis toxin-sensitive G<sub>i</sub> protein. In addition, S1P also acts as a chemoattractant for hematopoietic precursor cells and immature dendritic cells, raising the possibility that S1P may control the recruitment of inflammatory cells to sites of inflammation and help to modulate the immune response. Like S1P<sub>1</sub>, S1P<sub>3</sub> also appears to be involved in -stimulation of cell migration, -regulation of cytoskeletal rearrangements, -membrane ruffling associated with cell motility, and induction of neurite retraction and neuronal cell rounding. In addition to its intracellular anti-apoptotic role, binding of S1P to S1P<sub>3</sub> has also been shown to enhance survival by suppression of Bax expression and activation of endothelial nitric oxide synthase, phosphatidylinositol 3-kinase and Akt [133]. However, contrary to the stimulatory effects of S1P<sub>1</sub> and of S1P<sub>3</sub>, activation of S1P<sub>2</sub> inhibits cell migration. That S1P can both stimulate and inhibit cell migration at first appears contradictory, but the net effect of S1P on cell migration may depend on the relative levels of receptor expression, receptor turnover, and S1P concentrations. Indeed, at low concentrations, S1P induced smooth muscle cell migration but at higher concentrations it was inhibitory. Much less is known about the function of S1P<sub>5</sub>. It is, however, highly expressed in oligodendrocytes and astrocytes and S1P after binding with S1P<sub>5</sub> inhibits extracellular signal-regulated kinase (ERK) activation and proliferation in cells over-expressing S1P<sub>5</sub>. It is therefore

possible that S1P/S1P<sub>5</sub> signaling may play an important role in nervous system development [105].

Activation of a number of signaling pathways attributed to extracellular S1P may account for some of the observed biological effects [103]. Consistent with these observations, stimulation of these receptors results in either activation or inhibition of members of the Rho family of small GTPases, most prominently Rho and Rac. The activation of the small GTPases Rac and Rho has been linked to cytoskeletal rearrangements and motility. Activated Rho thus induces the formation of stress fibers, and activated Rac induces the formation of cortical actin network. Activation of S1PR<sub>1</sub> promotes this latter function of Rac, whereas activation of S1PR<sub>2</sub> inhibits it and thereby prevents Rac-induced chemotaxis and membrane ruffling. Other relevant S1P mediated signaling pathways include activation of the ERK and p38 mitogen-activated protein kinases, intracellular calcium mobilization, and activation of PLD and Akt. The differential expression of S1PRs is thus able to determine the chemotactic responses of cells to extracellular gradients of cells [133].

### **8.1.3 S1P as an intracellular second messenger**

In addition to its extracellular actions, S1P functions as a second messenger in the regulation of cellular proliferation, cell survival, Ca<sup>++</sup> homeostasis, and suppression of apoptosis. The intracellular target of S1P remains much more elusive. Although no direct targets have yet been conclusively identified, several lines of evidence support the second messenger role of S1P: 1) dihydro-S1P binds to and activates all of the identified S1PRs, but it does not mimic all of the effects of S1P, especially those related to cell survival, [143]; 2) yeast cells do not possess GPCRs, yet the abundance of phosphorylated long-chain sphingoid bases regulates environmental stress response, cell proliferation, and cell survival in a manner reminiscent of the function of S1P in mammalian cells; (3) in plants, which do not express S1PRs, S1P regulates Ca<sup>++</sup> homeostasis and ion channel [100]; and (4) microinjection of S1P into, or photolysis of cages S1P within, mammalian cells induces both Ca<sup>++</sup> mobilization and cell proliferation. An early clue that S1P may play a role as a second messenger mobilizing calcium from internal

sources, independently of inositol trisphosphate, arose with the finding that sphingosine derivatives generated inside cells stimulated the release of calcium.

S1P has also been shown to activate ERK and inhibit c-Jun N-terminal kinase (JNK) activation, which is significant since the balance of ERK and JNK activation has been implicated in the control of apoptosis. Moreover, ceramide, another sphingolipid metabolite that induces apoptosis in many cell types opposes the effects of S1P on these pathways. This, as well as the opposing effects of S1P and ceramide on the induction of apoptosis, has led to the model in which the dynamic balance of S1P and ceramide determines the fate of the cell [133]. More recently, elevated sphingosine levels in mast cells have been shown to inhibit allergic activation and production of leukotrienes, whereas elevated S1P levels resulted in activation of mast cells and increased leukotriene production, supporting the notion that the balance of intracellular sphingolipid metabolites controls many biological responses. As would be expected for an intracellular signaling molecule, the levels of S1P in cells are low and tightly regulated by the balance between sphingosine kinase-dependent synthesis and degradation by an endoplasmic pyridoxal phosphate requiring lyase and by phosphohydrolases. Recently, a specific S1P phosphohydrolase was cloned and characterized. Overexpression of this S1P-degrading enzyme decreased cellular S1P levels, increased sphingosine and ceramide, and promoted apoptosis, in further support of the model in which the dynamic balance of S1P and ceramide determines the fate of the cell. SPHK also plays an important regulatory role in the cardiovascular system. Activation of this enzyme and the consequent generation of S1P contribute both to the stimulation of endothelial cells by TNF- $\alpha$  and to the triggering of a signaling pathway by this cytokine that protects the cells against apoptosis [132]. The ability of HDL to inhibit the cytokine-induced expression of adhesion molecules in endothelial cells has also been correlated with resetting of the sphingolipid rheostat. This effect of HDL might be responsible, at least in part, for the protective function of this lipoprotein against the development of atherosclerosis and associated coronary heart disease [105, 133].

## 9. Tuberculosis therapeutics: past, present and future scenario

### 9.1 Brief history of anti-TB therapy

Archaeological evidence indicates that TB has afflicted the mankind for thousands of years and until about 50 years ago, this disease was considered virtually incurable. The discovery of several active anti-TB agents, however, beginning with streptomycin in the early 1943's, followed by several others, to the Rifampicin in the 1963, heralded a new age of anti-TB chemotherapy [17]. However, it became soon apparent that several factors such as long period of treatment, patients non-compliance, adverse side effects of therapeutic agents, the emergence of multi-drug resistant strains, and the concomitant HIV pandemic started representing an obstacle for the current therapies and hence eradication of the disease [128].

Soon after the identification of causative agent of TB, Koch created new methods to cultivate, MTB, “the tuberculin” that he thought initially to have therapeutically potentiality but rapidly showed inexistent (in-consistent). However, the tuberculin was shown special advantage to diagnostics of TB cases. Koch extracted the tuberculin from heat inactivated supernatant of MTB culture, grown in broth medium containing glycerol. In, 1905 Koch received the Nobel Prize for medicine for his studies on TB. Successively, he did an important observation that the tuberculin when administered subcutaneously in animals and human with TB, causes a particular reaction at 24-48 hours post-inoculation. This reaction, evident in epidermal level, was called as ‘the Koch’s phenomenon’ and today it is identified with the ‘DTH response’ and still a most common method for the laboratory diagnosis of tuberculosis. At the beginning of 1900, two French scientists, Albert Calmette and Camille Guérin, obtained an attenuated strain of *M. bovis*, which they proposed like an oral vaccine for human against TB. This vaccine called BCG and is responsible for most of false positivity result at the tuberculin’s test. Anyway, although it is now used like neonatal vaccine prevalently in developed country, to prevent the fulminant form of tubercular meningitis of children’s, it’s efficacy for pulmonary TB of adult is seriously in discussion. In the middle of 19<sup>th</sup> century, improved sanatorium conditions and providing adequate nutrition were

widely in use throughout Europe and later in the United States and were the only means to strengthen the immune system against this bacillus. In addition, from time to time several strategies such as collapse therapy, radiation, pneumothorax (artificial pneumothorax, bilateral pneumothorax), gold therapy, etc. were adopted to improve the condition of patients.

The hunt for the anti-TB drug partially ends on 1940 with the discovery of actinomycin with good *in vitro* anti-TB potential. However, this proved to be too toxic for use in humans or animals. Later, the success in the battle against TB came in the 1943, when streptomycin (SM) an aminoglycoside antibiotics, isolated from *Streptomyces griseus*, was became available in late 1944 as the first effective anti-TB drug [90]. It acts on protein synthesis machinery by binding with 30s subunit, decreases the fidelity of mRNA and garbles the message, leading to nonsense proteins. However, use of this drug alone, although initially effective, eventually resulted in a large number of patients with relapse due to development of resistance [26]. Fortunately, the efficacy of combination therapy was soon demonstrated, when a second drug para-aminosalicylic acid (PAS), a structural analog of p-aminobenzoic acid (PABA), used in combination with SM, reduced the occurrence of resistant strains [91]. But it was poorly tolerated [2]. The introduction of isoniazid (INH) in 1954 was a major breakthrough because of its potency. INH with SM and PAS proved even more effective at preventing the emergence of drug-resistant disease. The availability of ethambutol (EMB) subsequently led to the standard regimen for 18-24 months duration consisting of INH, SM, and EMB throughout the 1960s [92]. During this period, the concentration was on finding an effective intermittent regimen that would minimize development of resistance and shortening the course of chemotherapy. The availability of rifampicin (RFP) in the early 1963s inaugurated the modern era of TB chemotherapy [52]. The drug targets the DNA dependent RNA polymerase of the bacilli and enjoys high patient compliance and acceptability. The potent activity of RFP against the more slowly growing bacilli, along with the re-discovery of pyrazinamide (PZA) as an anti-tuberculous agent, allowed treatment regimens to be eventually shortened from 18 months to 6 months [48]. Presently several efforts are underway not only to reduce the time course of existing chemotherapy but also to improve the management of HIV co-infection and MDR-TB cases .

## 9.2 Existing therapy: chemotherapy

For the treatment of TB the existing chemotherapies are classified into two groups depending upon degree of effectiveness and potential side effects. However, treatment consideration for each patient's varies, mainly depending on severity and forms of disease, history of previous treatment and other clinical manifestations. The first group, composed of INH, RFP, PZA, and EMB, is also known as "first line therapy" because of their activity and favorable toxicity profile. The WHO recommended a "first line therapy" (in case of non-MDR) involving INH, RFP, PZA, EMB for two months as a initial intensive phase, and INH, RFP for the next four months as a continuation phase with pills taken daily [156]. Although there are slight differences in the recommendations by several agencies, such as US Centers for Disease Control and Prevention, American Thoracic Society, and the UK Joint Tuberculosis Committee of the British Thoracic Society, WHO and International Union Against Tuberculosis and Lung Disease, all recommended treatment regimens addressing i) the killing of actively growing and semi-dormant bacilli in the first initial intensive phase, and ii) the elimination of the last residual bacilli, to prevent failures, relapses and drug resistance, in the continuation phase.

The second group of agents are seldom used except in areas with high rates of drug resistance [66] or, as with thiacetazone, where low cost of drug is concern. This group of drugs includes, but is not limited to, fluoroquinolones (such as ofloxacin, ciprofloxacin), aminoglycosides (streptomycin), cycloserine, macrolides, glycopeptides, ethonamide, PAS, thiacetazone. However, the long-term effects of these drugs remain unknown due to the lack of research information. 1<sup>st</sup> and 2<sup>nd</sup> line drugs presently being used in the treatment of TB and some promising new developing anti-TB drugs are summarized, with their mode of action and major adverse effects, in the **table 2, 3, and 4** respectively.

**Table 2: Doses, route of administration, mode of action, and major adverse effects of primary drugs used in the treatment of tuberculosis.**

Generic Name	Effective Dose	Maximum Dose/Day	Route of Administration	Adverse Effects*	Mode of Action
Isoniazid	5mg/kg static for resting, cidal for growing -bacilli	300mg	Oral or IM	Hepatotoxic, Peripheral neuropathy, Arthralgia in elderly, Lupus like syndrome	Inhibits mycolic acid biosynthesis.
Rifampicin	8-12mg/kg cidal	600mg	Oral or IV	Hepatotoxic, Vomiting, Nausea, Anorexia	Inhibits DNA dep. RNA polymerase thus inhibits RNA synthesis.
Pyrazinamide	1.5-2.5g/kg cidal	-	Oral	Hepatotoxic, Hyperuricemia	Fatty acid synthase I.
Ethambutol	15mg/kg static 25mg/kg cidal	2.5g	Oral	Optic neuritis, Renal failure	Inhibits arabinan syn. both in AG and LAM
Streptomycin	20mg/kg cidal	1.0g	IM or IV	Ototoxicity, Renal dysfunction	Inhibits protein synthesis machinery.

**Table 3: Second line drugs used in the treatment of tuberculosis: doses, spectrum, and major adverse effects\*.**

Generic Name	Chemical Class	Maximum Dose/Day	Spectrum	Route of Administration	Major Adverse Effect
Ciprofloxacin, Ofloxacin, Levofloxacin	Fluoroquinolones	400-800mg 500-750mg	Bacteriostatic	Oral or IV	Fever, Rash, Abdominal cramps, Gastrointestinal upset, Insomnia
Cycloserine	Analogue of D-alanine	500-1000mg	Bacteriostatic	Oral	CNS (Somnolence) irritability, Psychosis, Depression, Rash
Capreomycin, Kanamycin, Amikacin	Aminoglycosides	1000mg	Bactericidal	IM, IV	Otic and Nephrotoxic, Hypokalaemia
Ethionamide, Prothionamide	Synthetic nicotinamide analogue	500-750mg	Bacteriostatic	Oral	Gastrointestinal upset, Hepatotoxic, Hypothyroidism
PAS	Structural analogue of PABA	8.0-12.0g	Bacteriostatic	Oral	Hepatotoxic, Hypersensitivity, Gastrointestinal upset
Rifabutin	Rifamycin	600mg	Bactericidal	Oral	Uveitis, Neutropenia, Rash, Hepatitis, Fever
Azithromycin, Clarithromycin	Macrolides	1.0g	Bacteriostatic	Oral or IV	Diarrhea, Vomiting, Rash, Abdominal pain, Headache
Vancomycin	Glycopeptides	1.0-2.0g	Bacteriostatic	IV	Nephrotoxicity, Rash, Auditory nerve damage

IV=Intravenous; IM=Intramuscular; AG=Arabinogalactan; LAM=Lipoarabinomannan; CNS=Central Nervous System; PAS= p-aminosakicylic acid; PABA= p-aminobenzoic acid. \*Not all toxicities are listed here. Full prescribing information should be checked in the product insert or pharmacology texts. In order to see the details of each individual drug, drug reference manuals are recommended.



**Table 4: Promising newly developing anti-mycobacterial agents [reviewed in 51]**

Agents	Stage of Development	Advantages	Disadvantages
Thiolactomycine & Analogues	Discovery	<ol style="list-style-type: none"> <li>1. Good <i>in vitro</i> anti -MTB activity.</li> <li>2. Synthetic routes to thiolactomycine and analogue is available.</li> <li>3. Like INH act on FAS II, with no cross interaction.</li> <li>4. Unlike INH, does not require KatG for <i>in vivo</i> activation, hence active against INH resistant strains.</li> <li>5. Well absorbed orally.</li> </ol>	<ol style="list-style-type: none"> <li>1. No <i>in vivo</i> report against MTB is available.</li> <li>2. Parent molecule is chiral, cost implications if analogues are also chiral.</li> </ol>
Ethambutol & Analogues (NIH 241)	Discovery	<ol style="list-style-type: none"> <li>1. More potent <i>in vivo</i> than EMB.</li> <li>2. Likely to have less toxicity than EMB.</li> </ol>	<ol style="list-style-type: none"> <li>1. Like EMB cytostatic action.</li> <li>2. Parent molecules is chiral, cost implications is possible.</li> </ol>
Mefloquine & derivatives WR-3016, WR-3017, desbutylhalofantrine)	Discovery	<ol style="list-style-type: none"> <li>1. Good <i>in vitro</i> activity vs MTB and MAC.</li> <li>2. Many analogues available.</li> </ol>	<ol style="list-style-type: none"> <li>1. Non apparent but possibility of strong protein-binding should be checked.</li> </ol>
Diazpteridines & analogues (SRI-20094, NJ 3440)	Discovery	<ol style="list-style-type: none"> <li>1. 20094 selectively inhibits mycobacterial dihydro folate reductase.</li> <li>2. 3D structure of mycobacterial MDRF is known, which allow the rational synthesis for other inhibitors.</li> <li>3. Potentially useful for <i>M. avium</i> infection in AIDS patients.</li> </ol>	<ol style="list-style-type: none"> <li>1. <i>In vivo</i> information is lacking.</li> </ol>
9-Benzyl purines & derivatives	Discovery	<ol style="list-style-type: none"> <li>1. Good <i>in vitro</i> anti -MTB activity with low cellular toxicity.</li> <li>2. Structurally dissimilar to existing TB drugs and hence cross-resistance unlikely.</li> </ol>	<ol style="list-style-type: none"> <li>1. <i>In vivo</i> data remain to determine.</li> <li>2. No information on mode of action.</li> </ol>
Benzoxazines & analogues	Discovery	<ol style="list-style-type: none"> <li>1. Lead compound have good INH comparable <i>in vitro</i> activity against spectrum of mycobacterial species.</li> <li>2. Range of analogues available.</li> </ol>	<ol style="list-style-type: none"> <li>1. No <i>in vivo</i> data yet available.</li> <li>2. Mode of action not known.</li> <li>3. Possible cross-activity with thiaacetazone due to presence of thioamide moiety.</li> </ol>
Tryptanthrin & Analogues	Discovery	<ol style="list-style-type: none"> <li>1. Active against drug resistant strains.</li> <li>2. Moderate <i>in vivo</i> activity.</li> </ol>	<ol style="list-style-type: none"> <li>1. Lack of toxicity data.</li> </ol>
Tetramethylpiperidin (TMP) phenazines & analogues (B4169, B4128)	Discovery	<ol style="list-style-type: none"> <li>1. Potent activity against MDR strains.</li> <li>2. Active intra- and extra-cellularly.</li> <li>3. Superior activity in comparison to both clofazimine and rifampicin.</li> </ol>	<ol style="list-style-type: none"> <li>1. No <i>in vivo</i> data.</li> <li>2. Mode of action not known.</li> <li>3. Coloration of skin is likely a problem.</li> </ol>

Quinolones (PD 161148, CS-940)	Preclinical	<p>1. Potent bactericidal and synergizes with other fluoroquinolones.</p> <p>a) CS-940 +SPFX (IC<sub>50</sub> of 0.25- 0.5 µg/ml) &gt; +OFLX = +CFLX &gt; (IC<sub>50</sub>s of 0.5 to 2.0 µg/ml) &gt;NFLX (IC<sub>50</sub>s of 8 to 16 µg/ml).</p> <p>b) PD 161148 was found to be 3-4 folds active in comparison to desmethoxy analogue, CFLX</p>	<p>1. No <i>in vivo</i> and toxicity data available.</p> <p>2. From structural consideration, the molecules do not appear to be too metabolically robust.</p>
2-Pyridone (ABT-255)	Preclinical	<p>1. <i>In vitro</i> and <i>in vivo</i> more efficient than RFP or EMB vs drug-sensitive and resistant strains.</p> <p>2. No cross resistance with quinolones and existing drugs.</p>	<p>1. Don't sterilize mouse lung tissue, suggesting its use as supplement rather than replacement.</p> <p>2. Toxicity studies lacking.</p>
Calanolide -A, -B & -13 other pyranocoumarin	Preclinical	<p>1. Possible dual role in treating TB and AIDS, as it shows <i>in vitro</i> good anti- MTB activity and inhibits HIV-1 RT.</p> <p>2. Synthetic route developed to calanolide A and analogues; however calanolide B is readily obtainable from renewable natural sources.</p>	<p>1. For TB medication, no <i>in vivo</i> efficacy and toxicity profile is available in public domain</p>
Nitroimidazoles & analogues (PA 824, PA 1343, CGI 17341)	Preclinical	<p>1. Potent cidal activity against drug-sensitive and -resistant strains of MTB (CGI 17341, 0.04-0.3; PA 824, 0.015-0.25; PA 1343, 0.015µg/ml respectively).</p> <p>2. Active even against dormant bacilli.</p> <p>3. Oral bioavailability and least toxicity are appreciable.</p> <p>4. No any cross-resistance with existing TB drugs.</p>	<p>1. Presence of the nitro moiety in these compounds raises concerns about the possibility of geno-toxicity and this issue remains to be addressed.</p>
Oxazolidinones (Linezolid U-100766, PNU 100480, DuP-721, AZD 2563)	Preclinical	<p>1. Very potent against drug -sensitive (0.125 µg/ml) as well as -resistant (2 µg/ml) strains.</p> <p>2. Novel mode of action, not interferes with other inhibitors of protein synthesis machinery</p> <p>3. Linezolid is available in the market.</p>	<p>1. No TB data for AZD 2563 is yet present.</p> <p>2. Toxicity data is still lacking for PNU.</p>
Phenazinamine derivatives (riminophenazines, B4157, B746, B4154)	Preclinical	<p>1. <i>In vitro</i> MIC<sub>90</sub> (MTB) 0.12 µg/ml; <i>in vivo</i> 20 mg/kg (C57BL/6 mice).</p> <p>2. Less skin pigmentation in respect to other compound of this group.</p> <p>3. Rapid localization within phagocytes.</p>	<p>1. No definitive toxicity data.</p> <p>2. Colorization of skin is likely to be problematic.</p> <p>3. Immunosuppressive properties compounds.</p>
Nicosamide	Preclinical	<p>1. Potent cidal to dormant and drug-resistant MTB.</p>	<p>1. Mutagenic and has reproductive toxicity.</p>
Pyrazinamide analogues (Pyrazinoic acid esters)	Preclinical	<p>1. <i>In vitro</i> more potent than PZA vs MTB.</p> <p>2. 900 fold greater serum stability respect to the compound of series.</p>	<p>1. Definitive <i>in vivo</i> data is still lacking.</p> <p>2. Cross-resistance with other drugs is likely.</p>
Quinolones & analogues a. Ciprofloxacin, Ofloxacin  b. Moxifloxacin, (BAY12-8039), Gemifloxacin (SB-	Clinical	<p>1. Being used as second-line or alternative anti-TB agents.</p> <p>2. Potent cidal against various clinical isolates with MIC<sub>90</sub> 0.25 and 8µg/ml respectively.</p> <p>3. Excellent oral bioavailability, safe and well</p>	<p>1. Development of resistance to these drugs.</p> <p>2. No clinical data for TB indication.</p>

265805)		<p>tolerated.</p> <p>4. Long <math>T_{1/2}</math> (12 and 8 hrs respectively) emerges the possibility of ONCE-a-day administration.</p> <p>5. Potent 4<sup>th</sup> generation quinolones.</p> <p>6. Comparable <i>in vivo</i> activity with low chances of resistance development.</p>	
Rifamycin & derivatives Rifameterane (SPA-S-565)	Clinical	<p>1. Equipotent to RFP vs MTB, but better pharmacokinetic profile</p> <p>2. Excellent oral bioavailability, safe and well tolerated.</p> <p>3. Elimination half life and mean resistance time in comparison to RFP 5-fold higher.</p>	<p>1. Cross resistance with RFP.</p> <p>2. No clinical data in public domain.</p>
Mikosome	Clinical	<p>1. Parent drug is already in use as second line drug vs MTB.</p> <p>2. Very effective <i>in vitro</i> and <i>in vivo</i> vs <i>M. avium</i> infections.</p> <p>3. Also effective against MTB in limited phase II clinical trial.</p> <p>4. 7-fold higher peak plasma level compared to free drug (amikacin).</p>	<p>1. Administration is either by iv route or aerosol.</p> <p>2. Liposomal preparation is expensive and stability may be of concern to address.</p>
Aconiazid	Clinical	<p>1. Pro-drug is well known anti -TB drug (INH)</p> <p>2. Lacks carcinogenicity and lower toxic than parent drug.</p>	<p>1. Cost will be higher than INH.</p> <p>2. Cross- resistance with INH.</p>
SRL-172	Clinical	<p>1. Immunenhancing approach containing heat killed <i>M vaccae</i> comprises Th1 adjuvant with bacterial antigens.</p> <p>2. Patients received this preparation along with routine chemotherapy shown reduced sputum smear positivity AFB, weight gain, and became afebrile earlier in respect to drug treatment alone.</p> <p>3. Safe, well tolerated and probably cost effective.</p>	<p>1. Unimpressive and inconsistent clinical data.</p> <p>2. Administration is not oral but by iv route.</p>

### 9.3 Problems associated with TB control

The main problem with TB treatment and preventing antibiotic resistance is patient's non compliance [66]. The treatment regimens have decreased in length from two years to six months but this is still a long time to take daily antibiotics. Moreover, due to the symptomatic improvement after one month of treatment, patients may then believe that they can stop therapy, and as a consequence, bacterial relapse may occur after some weeks, with high possibility to develop antibiotic resistance [66]. Finally, if the treatment does not continue for adequate length

of time at correct doses, mal-absorption of antibiotics can also occur [97]. These problems can be corrected by ensuring that the correct antibiotic regimen for adequate duration is followed and that regular susceptibility testing is carried out. The problem of non-compliance is complicated, as several studies have shown that reliable prediction of which patients will take all prescribed medication by themselves is not possible. A directly observed therapy short-course (DOTS) recommended by WHO in 1990 [156], is the most effective solution to reduce non-compliance generated problems amongst TB patients, as it involves health care workers actually witnessing and recording daily therapy, ensuring no dose can be missed and monitoring for possible emerging drug resistance problems. In brief, the recommended DOTS scheme has five main elements: i) political commitment, ii) early diagnosis by sputum smear microscopy, iii) direct observation short course treatment with effective case management, iv) a regular, uninterrupted supply of all essential anti-TB drugs, and, v) a standardized recording and reporting system.

One of the consequences of patient non-compliance or incorrect prescribing is the emergence of MDR-TB strains [66], which are defined if bacilli isolated are resistant to at least INH and RFP. The treatment of MDR-TB cases remains extremely difficult and requires meticulous laboratory studies to characterize the susceptibility of these isolates to other drugs. In fact, contrary to the first-line drugs, which can be easily screened *in vitro*, based on previously established critical concentrations, such information's are unavailable for most of the second-line and/or newer anti-tuberculous drugs [66]. Moreover, considering the incidence of HIV infection in TB patients, most of the drugs used in TB or MDR-TB have not received drug/drug interaction studies performed with antiviral compounds, and the potential for adverse inter-reactions may be considerable. In this context, the longer acting rifamycin; rifapentine, received in 1998, approval by the FDA in the USA as an anti-TB drug, but was found to interact significantly with the AIDS drug indinavir [66]. Finally, without good public health policy implemented inside and outside institutions like hospitals, the potential for large MDR-TB outbreaks among immunosuppressed patients remains a frightening reality. In order to combat the emerging epidemic of MDR-TB, WHO is conceived a DOTS-Plus, which is a comprehensive management strategy

under continuous development and testing, that includes the tenets of the DOTS strategy. DOTS-Plus takes into account specific issues (such as the use of second-line anti-TB drugs) that need to be addressed in areas where there is high prevalence of MDR-TB [155].

TB epidemic both in developed and developing countries before the 1980, with recommended four drug regimen follow up was believed to be under control. However, since the 1980's the disease has been undergoing a resurgence, driven by i) a variety of changes in social, medical, economic factors, ii) a dramatic increase in immuno-suppressed individuals due mainly to HIV pandemic, [128] and iii) the occurrence of MDR mycobacterial strains. Such epidemiologic evolution has not yet been adequately contrasted by new efficacious drug [51]. In fact, it has been quite long since we have had any new efficacious anti-TB agents with least toxic profile. The last R&D breakthrough in TB drug development has occurred about forty years ago when the RFP was introduced in the 1960s. Several chemotherapeutical approaches after the discovery of MTB as an etiological agent of TB, has been applied in the past, but none hold the ground satisfactory to combat the present persisting problem and in fact, it has been quite long since we have had any new efficacious anti-TB agents with least toxic profile. This long gap in the search of efficacious anti-TB agent arguing the need of newer therapeutics, including novel chemotherapeutics agents and immune-modulators together with conventional drug regimen may shorten the course of therapy effectively and may manage MDR subjects as well

#### **9.4 Immune-modulators: an approach toward anti-TB therapy**

Resurgence of drug resistance strains, fatal side effects, treatment consideration in immune-suppressed subjects, need to lessen the duration of therapy, have induced to search for some alternative TB therapeutics. To this regard, utilization of chemotherapy along with immune-modulators is a bidirectional approach to fight against TB, where antibiotic regimen will directly target the bacterial population whereas immune-modulators will potentiate the effector anti-microbial mechanism not only to eradicate the pathogen in short time but also to reduce the involved tissue damage. Several efforts with conflicting results have been reported in the past in

the use of various cytokines such as IFN- $\gamma$ , INF- $\alpha$ , IL-2, IL-12, IL-18, CSF-1, GM-CSF, TNF- $\alpha$  [24, 34, 44, 46, 124, 138]. The role of IL-12, IL-18 in shifting of specific immune response towards Th1 and the importance of IFN- $\gamma$  in protection has been equivocally accepted [46, 124]. Simultaneously, some studies at use of these cytokines reported opposite results, such as those observed with CSF-1, where increase of intracellular growth of bacilli was found [34]. Other studies have shown that aerosolic administration of IFN- $\gamma$  to MDR-TB patients may be beneficial [24]. Moreover, IL-2 or GM-CSF has been recently found to be efficacious to some extent in improving patients with TB or disseminated MAC infection [138]. However, the direct uses of these cytokines, including INF- $\gamma$  and IL-12, as therapeutic agents against mycobacterial infections, appear to be limited, because of the possible induction of both immunosuppressive cytokines during adjuvant therapy and, in some cases, severe side effects. Thus, the development of new classes of immune-modulators other than cytokines, particularly those with no/least side effects, is needed. Several lines of evidence have indicated that vitamin D3 (1, 25-dihydroxyvitamin D3, calcitriol) regulates host resistance to MTB [34, 113], as Vitamin D3 deficiency and Vitamin D3 receptor polymorphisms have been linked to increased susceptibility to MTB and *M. leprae* infection [115, 152]. To this regard, *in vitro* use of Vitamin D3 has been shown to suppress the growth of MTB in various cell types (macrophage like HL-60 cell line, monocytes), at least partly through an NO-dependent mechanism [32, 113]. Moreover, experiments with recombinant cytokines and vitamins D3 showed that a combination of IFN- $\gamma$ , TNF- $\alpha$  and calcitriol induced a significant amount of intracellular killing of MTB [34]. However, *in vivo* promising results and involved molecular bactericidal mechanisms are still lacking for the possible therapeutic use of this molecule. Moreover, Cadranet et al. advocated that Vitamin D3 production by alveolar immune cells impaired the calcium metabolism in TB patients [13]. Since Ca<sup>++</sup> is an important factor in macrophage anti-mycobacterial activity [41, 87], further studies are required before its clinical use. *In vitro* treatment of human macrophages with ATP has recently been demonstrated to stimulate the killing of intracellular mycobacteria including various virulent strains of MTB [41, 77, 80]. This effect is mediated by purigenic P2Z

(P2X7) receptors, requires activation of host PLD and is often associated with rapid cell death [80]. However, the relevance of this approach in *in vivo* model remains to be determined.

Recently, it has been described that the stimulation of TLR-2 by the 19-kDa mycobacterial cell wall lipoprotein strongly activates antimicrobial activity in both murine and human macrophages [137]. Similarly, other bacterial products, such as bacterial DNA, characterized by unmethylated CpG motifs (CpG-ODN), known to bind with TLR-9, have been previously described to induce ROI [150] and NO [53] production in murine macrophages, and describe to increase antimicrobial activity against intracellular pathogens such as *Listeria monocytogenes* [76], *Leishmania major* [147], and mycobacterial species [60, 71]. Some mechanisms underlying the activation of host mycobactericidal activity such as induction of Th1 response [71] and PLD activation [6] have been reported. Recent experimental data regarding CpG induced an effective anti-viral immune response in Atlantic salmon [70] and possible use of CpG as a novel therapeutic tool for the treatment of atopic asthma [68], allergy [11] may represent a promising strategy for future. However, much attention has to be taken in the utilization of CpG before to start with clinical trials, as it may initiate inappropriate immune response and it has been reported to induce septic shock in murine model and to prime mice for Shwartzman reaction [25]. Small molecular weight modulators, which could be expected to be significantly cheaper and suitable for oral administration, have also shown preliminary promise. These compounds such as thalidomide, which blocks TNF- $\alpha$  production, have been reported to have beneficial effects on weight gain in both HIV-positive and -negative TB patients [139]. Other small molecular weight immune-modulators such as tucaresol, induces in mice cytotoxic memory T cells to kill MTB in infected cells, might also be useful, [109]. Encouragement should be provided to these pharmas to evaluate the potential of the compounds for TB therapy.

Recently reported results highlighted the possible contribution of some bioactive lipids in the protection against TB [4]. In this context, in the present study i utilized the S1P, a product of sphingolipid metabolism, to strengthen the host innate immunity against tuberculosis.

## Aims and Objectives

With such worst epidemic looming large on the earth, we urgently need new diagnostic and therapeutic intervention to combat this worldwide public health problem. The currently available remedies for fighting tuberculosis are inadequate. In the past decade, however, significant technological advances for discovering new, more selective and effective anti-TB agents have been made. The mapping of the MTB genome [23]; the delineation of many of the pathways in mycobacterial cell wall biosynthesis (e.g., glycosylation pathways, fatty acid biosynthesis, and diaminopimelic acid biosynthesis) [9, 12, 74]; and in mycobacterial metabolism, the discovery of genes involved in latency and virulence [9, 12]; application of DNA microarray technology [10, 54]; application of combinatorial chemistry and high-throughput screening to anti-TB drug discovery; and proteomic approach for target selection and drug mechanism of action studies have led to a fundamentally new paradigm in the pursuit of novel anti-mycobacterial agents.

On these backgrounds, present study addresses: i) whether human macrophages are able to control the intracellular replication of mycobacteria, if yes, then until what extent? ii) The role of human macrophage PLD in its differential ability to control intracellular replication of pathogenic and non-pathogenic mycobacteria such as MTB and *M. smegmatis* (Msm).

Moreover, as S1P has been shown to activate PLD, its efficacy as a novel regulator of anti-mycobacterial innate immunity has been *in vitro*, *in vivo* and *ex vivo* analysed.

Finally, in order to identify the S1P induced macrophages molecules and its biochemical pathway involved in mycobacterial killing a preliminary proteomic analysis have been performed.



# Materials and Methods

## 1. Material

### 1.1 Reagents

RPMI 1640, fetal bovine serum (Gibco, Invitrogen corporation, Paisley, UK), 7H9- and 7H10- Middle Brook, dextrose (Difco, Becton Dickinson, France S.A.), L-glutamine, phosphate buffer saline, tris, sodium chloride, di sodium hydrogen phosphate, mono sodium di hydrogen phosphate, potassium chloride, glycine, SDS, urea, thio-urea, ampicillin, catalase, sucrose, imidazole, acrylamide, bisacrylamide, ammonium per sulphate, TEMED, Tween-20, Tween-80, Triton X-100, oleic Acid, phosphatidylcholine, phospholipase D, Phenyl methyl sulfonyl fluoride, iodoacetamide, di thio threitol, bromophenol blue, coomassie blue, agarose, poly-L-lysine (Sigma, St. Louis, Mo), non-essential aminoacids, sodium pyruvate, gentamycine (GibcoBRL, Life Technologies, Paisley, UK), fluconazole (Pfizer), anti-lysosomal associated membrane protein-1, anti-cathepsin D (BD transduction laboratories), anti-PLD (Quality controlled Biochemicals, Hopkinton, MA, USA), ethanol, methanol, chloroform, acetic acid, propanol, butanol, isobutanol, formaldehyde (formalin), (Chemica Fluka, GmbH, Germany ), albumin bovine Serum, sphingosine 1-phosphate, sphingosine dihydro, sphingosine 1-phosphate di hydro, glycerol, leupeptin, aprotinin, pepstatin, (Calbiochem, CA, USA), Lysotracker Red (Molecular Probe), Auramine kit (BD Microbiology Systems, Sparks, MA, USA), non fat dry milk, goat anti-human-IgG antibodies (Bio-Rad laboratories), Micro BCA<sup>TM</sup> Protein Assay Reagent Kit, SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL), silver Staining kit for protein, IEF preservative solution, IEF strips, rainbow molecular weight standard (Amersham Biosciences), silica gel plates (Merck), pretreated slides (VWR International Merck Euro lab, FR), vectashield mounting medium H-1000 (Vector laboratories, CA).

### 1.2 Media

I. RPMI 1640 complete medium	for macrophages	for THP1 cells
RPMI 1640	500ml	500ml
FCS	10%	10%
L-Glutamine	5μM	5μM

Non-essential aminoacids	-	1 $\mu$ M
Sodium Pyruvate	-	1 $\mu$ M
Gentamycine	5 $\mu$ g/ml	5 $\mu$ g/ml

## II. 7H9 Middle Brook supplemented with ADC

1. To prepare 1 liter of medium, suspend 4.7 gm 7H9 middle brook dehydrated powder in 850 ml of d H<sub>2</sub>O. Add 2 ml glycerol, and make up the volume with dH<sub>2</sub>O up to 900 ml. Heat to dissolve completely.
2. Sterilize in autoclave for 10 minutes at 15 lbs pressure (121°C).
3. Cool to 50-55°C, and then add aseptically 100 ml of the ADC (see appendix for preparation) enrichment at final concentration of 10% and mix thoroughly.
4. Aliquot aseptically as desired.

## III. 7H10 Middle Brook supplemented with OADC

1. To prepare 1 liter of final medium, suspend 19 gm 7H10 middle brook dehydrated powder in 850 ml of dH<sub>2</sub>O. Add 5 ml glycerol, and make up the volume with dH<sub>2</sub>O up to 900 ml. Heat to dissolve completely.
2. Sterilize in autoclave for 10 minutes at 15 lbs pressure (121°C).
3. Cool to 50-55°C, and then add aseptically 100 ml of the OADC (see appendix for preparation) enrichment at final concentration of 10% and mix thoroughly.
4. Dispense in Petri plates as desired.

IV. Enrichment for Middle Brook medium	ADC	OADC
Oleic acid	-	0.05 g
Albumin fraction V bovine	5.0 g	5.0 g
Dextrose	2.0 g	2.0 g
Catalase (Beaf)	0.003 g	0.004 g
Sodium Chloride	-	0.85
Distilled H <sub>2</sub> O upto	100 ml	100 ml

Dissolve all the ingredients and filter aseptically through a 0.2 mm filter. Store at 4°C.

### 1.3 Buffers and Solutions

#### A. Phosphate Buffer Saline (PBS) 150mM pH 7.2<sup>1</sup>

(140 mM NaCl, 20 mM KCl, 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 20 mM KH<sub>2</sub>PO<sub>4</sub>, ddH<sub>2</sub>O)

	Final Concentration	Amount
NaCl (FW 58.44)	140 mM	8 g
KCl (FW 136.1)	20 mM	2 g
Na <sub>2</sub> HPO <sub>4</sub> (FW 142.0)	50 mM	11.5 g
KH <sub>2</sub> PO <sub>4</sub> (FW 136.1)	20 mM	2.0 g
Double distilled H <sub>2</sub> O		1000 ml

<sup>1</sup> Before make up the volume 1000 ml with ddH<sub>2</sub>O, check and adjust the pH to 7.2.

Filter solution through a 0.45 mm filter. Store at 4°C.

#### B. SDS lysis buffer<sup>1</sup>

(2% SDS, 40 mM Tris base, 100 mM NaCl, ddH<sub>2</sub>O)

	Final Concentration	Amount
SDS (FW 288.38)	2% (w/v)	0.8 g
Tris base (FW 121.1)	40 mM	0.194 g
NaCl (FW 58.44)	100 mM	0.16 g
Double distilled H <sub>2</sub> O		to 40 ml

Prepare fresh or store in aliquots at -20°C

<sup>1</sup> Protease inhibitors (1mM PMSF, 2000 iu/ml Apotinin, 100 µM Leupeptin, 50 µM Pepstatin A) and/or reductants (DTT 60 mM) is added just prior to use.

#### C. Urea lysis solution<sup>1</sup>

(8 M Urea, 4% CHAPS, 40 mM Tris base, ddH<sub>2</sub>O)

	Final Concentration	Amount
Urea (FW 60.06)	8 M <sup>2</sup>	19.2 g
CHAPS <sup>3</sup>	4% (w/v)	1.6 g
Tris base (FW 121.1)	40 mM	0.194 g
Double distilled H <sub>2</sub> O		to 40 ml

Prepare fresh or store in aliquots at -20°C

<sup>1</sup> Protease inhibitors ((1mM PMSF, 2000 iu/ml Apotinin, 100 µM Leupeptin, 50 µM Pepstatin A) and/or reductants (DTT 60 mM) is added just prior to use.

<sup>2</sup> If necessary, the concentration of urea can be increased to 9 or 9.8 M.

<sup>3</sup> Other detergents (Triton X-100, NP-40, and other non ionic or zwitterionic detergents) can be used instead of CHAPS.

#### **D. Rehydration stock solution without IPG buffer<sup>1</sup>**

(8 M Urea, 2% CHAPS, Bromophenol Blue, ddH<sub>2</sub>O)

	<b>Final Concentration</b>	<b>Amount</b>
Urea (FW 60.06)	8 M <sup>2</sup>	12 g
CHAPS <sup>3</sup>	2% (w/v)	0.5 g
Bromophenol blue	trace	(a few grains)
Double distilled H <sub>2</sub> O		to 25 ml

Store in 2.5 ml aliquots at -20°C

<sup>1</sup> DTT and IPG buffer are added just prior to use: Add 7 mg DTT per 2.5 ml aliquot of rehydration stock solution. In order to have required concentration (0.5-2%); add appropriate volume of IPG buffer to it. If loading sample by inclusion in the rehydration solution, sample is also added to the 2.5 ml of rehydration solution just prior to use.

<sup>2</sup> If necessary, the concentration of urea can be increased to 9 or 9.8 M

<sup>3</sup> Other detergents (Triton X-100, NP-40, and other non ionic or zwitterionic detergents) can be used instead of CHAPS.

#### **E. Rehydration stock solution with IPG buffer<sup>1</sup>**

(8 M Urea, 2% CHAPS, 0.5% or 2% IPG buffer<sup>2</sup>, Bromophenol Blue, ddH<sub>2</sub>O)

	<b>Final Concentration</b>	<b>Amount</b>
Urea (FW 60.06)	8 M <sup>3</sup>	12 g
CHAPS <sup>4</sup>	2% (w/v)	0.5 g
IPG buffer	0.5% or 2% (v/v)	125 or 500 µl <sup>5</sup>
Bromophenol blue	trace	(a few grains)
Double distilled H <sub>2</sub> O		to 25 ml

Store in 2.5 ml aliquots at -20°C

<sup>1</sup> DTT and IPG buffer are added just prior to use: Add 7 mg DTT per 2.5 ml aliquot of rehydration stock solution. If loading sample by inclusion in the rehydration solution, sample is also added to the 2.5 ml of rehydration solution just prior to use.

<sup>2</sup> Either of two IPG buffer concentrations is recommended (Amersham Biosciences). Selection of IPG buffer concentration is based on the IEF system and the pH range of the IPG strip.

<sup>3</sup> If necessary, the concentration of urea can be increased to 9 or 9.8 M

<sup>4</sup> Other detergents (Triton X-100, NP-40, and other non ionic or zwitterionic detergents) can be used instead of CHAPS.

<sup>5</sup> Use 125 µl of IPG buffer for a 0.5% concentration and 500 µl IPG buffer for a 2% concentration.

#### F. SDS equilibration buffer<sup>1</sup>

(50 mM Tris-Cl pH 8.8, 6 M Urea, 30% glycerol, 2% SDS, bromophenol blue, ddH<sub>2</sub>O)

	Final Concentration	Amount
1.5 M Tris-Cl pH 8.8	50 mM	6.7 ml
Urea (FW 60.06)	6 M	72.07 g
Glycerol (87% v/v)	30% (v/v)	69 ml
SDS (FW 288.38)	2% (w/v)	4.0 g
Bromophenol blue	trace	(a few grains)
Double distilled H <sub>2</sub> O		to 200 ml

Store in 20 ml aliquots at -20°C

<sup>1</sup> This is a stock solution. Prior to use add 100 mg of DTT per 10 ml of stock solution is added. In a separate 10 ml of stock solution 250 mg of iodoacetamide is added.

#### G. Monomer stock solution

(30% acrylamide, 0.8% N,N'-methylenebisacrylamide, ddH<sub>2</sub>O)

	Final Concentration	Amount
Acrylamide (FW 71.08)	30%	60.0 g
N,N'-methylenebisacrylamide (FW 154.17)	0.8%	1.6 g
Double distilled H <sub>2</sub> O		to 200 ml

Filter solution through a 0.45µ filter. Store at 4°C away from light.

#### H. 4X Resolving gel buffer

(1.5 M Tris-Cl pH 8.8, ddH<sub>2</sub>O)

	Final Concentration	Amount
1.5 M Tris base (FW 121.1)	1.5 M	181.5 g
Double distilled H <sub>2</sub> O		750 ml
HCl (FW 36.46)		adjust pH to 8.8
Double distilled H <sub>2</sub> O		to 1000 ml

Filter solution through a 0.45 $\mu$  filter. Store at 4°C.

#### I. 4X Stacking gel buffer

(0.5 M Tris-Cl pH 6.8, ddH<sub>2</sub>O)

	Final Concentration	Amount
0.5 M Tris base (FW 121.1)	0.5 M	6.05 g
Double distilled H <sub>2</sub> O		40 ml
HCl (FW 36.46)		adjust pH to 6.8
Double distilled H <sub>2</sub> O		to 100 ml

Filter solution through a 0.45 $\mu$  filter. Store at 4°C.

#### J. 10% SDS

(SDS, ddH <sub>2</sub> O)	Final Concentration	Amount
SDS (FW 288.38)	10% (w/v)	10.0 g
Double distilled H <sub>2</sub> O		to 100 ml

Filter solution through a 0.45 $\mu$  filter. Store at room temperature.

#### K. 10% Ammonium persulphate (APS)

(APS, ddH <sub>2</sub> O)	Final Concentration	Amount
Ammonium persulphate (FW 228.20)	10% (w/v)	0.1 g
Double distilled H <sub>2</sub> O		to 1.0 ml

Fresh APS "crackles" when water is added. If it does not, replace it with fresh stock.

Prepare just prior to use.

#### L. Gel storage solution

(0.375 M Tris-Cl pH 8.8, 0.1% SDS, ddH<sub>2</sub>O)

	Final Concentration	Amount
4X resolving gel buffer	1 X	50 ml
10% SDS	0.1%	2 ml
Double distilled H <sub>2</sub> O		to 200 ml

Store at 4°C.

#### M. SDS electrophoresis buffer<sup>1</sup>

(25 mM Tris, 192 mM glycine, 0.1% SDS, ddH<sub>2</sub>O)

	Final Concentration	Amount
Tris base (FW 121.1)	25 mM	15.1 g
Glycine (FW 75.07)	192 mM	72.1 g
SDS (FW 288.38)	0.1% (w/v)	5.0 g
Double distilled H <sub>2</sub> O		to 5000 ml

Store at room temperature.

<sup>1</sup>Because the pH of this solution need not to be checked, it can be made up directly in large reagent bottles marked at 5.0 liters, Or, it can be made as 5X solution by taking the amount of all component five times more than its original.

#### N. Agarose sealing solution

(25 mM Tris, 192 mM glycine, 0.1% SDS, 0.5% agarose, bromophenol blue, ddH<sub>2</sub>O)

	Final Concentration	Amount
SDS electrophoresis buffer		100 ml
Agarose (NA or M)	0.5%	0.5 g
Bromophenol blue	trace	(a few grains)

Add all ingredients into 500 ml conical flask. Swirl to disperse. Heat in a microwave oven on low until the agarose is completely dissolved. Do not allow the solution to boil over. Dispense 2 ml aliquots into screw-cap tubes and store at room temperature.



#### O. Western blotting (WB) transfer buffer<sup>1</sup> (pH 9.2)<sup>2</sup>

(48 mM Tris, 39 mM glycine, 20% methanol, 1.3 mM SDS (0.0375%), ddH<sub>2</sub>O)

	Final Concentration	Amount
Tris base (FW 121.1)	48 mM	5.82 g
Glycine (FW 75.07)	39 mM	2.93 g
SDS (FW 288.38)	1.3 mM (0.0375%) (w/v)	0.375 g
Methanol (analytical reagent grade)	20% (v/v)	200 ml
Double distilled H <sub>2</sub> O		to 1000 ml

Prepare always fresh and put at 4°C prior to use.

<sup>1</sup>The above western blotting transfer buffer recipe is based on the protocol utilized by Bjerrum and Schafer-Nielsen which has been recommended for transfer of SDS-protein using nitrocellulose membrane with the trans-blot SD cell.

<sup>2</sup>Do not add acid or base to adjust pH. The buffer will range from pH 9.0 to 9.4, depending on the quality of the reagents.

#### P. Western blot (WB) wash buffer

(150 mM PBS pH 7.2, 0.1% Tween-20)

	Final Concentration	Amount
PBS (see appendix) pH 7.2	150 mM	1000 ml
Tween-20	0.1% (v/v)	1.0 ml

#### Q. Western blot blocking buffer

(150 mM PBS pH 7.2, 0.1% Tween-20, 5% Non fat dry milk)

	Final Concentration	Amount
PBS (see appendix) pH 7.2	150 mM	100 ml
Tween-20	0.1% (v/v)	0.1 ml
Non fat dry milk powder <sup>1</sup>	5%	5 g

<sup>1</sup>Non fat dry milk can be replaced with the appropriate concentration of BSA, or normal sera as per the required protocol.

## R. Silver staining solutions (Amersham Biosciences protocol)<sup>1</sup>

(Fixing solution, Sensitizing solution, Silver solution, Developing solution, Stop solution, Gel preserving solution)

<sup>1</sup> Freshly prepares solutions give best results.

### a. Fixing solution

(50% ethanol, 10% acetic acid, dH<sub>2</sub>O)

	Stock concentration	Final concentration	Amount
Ethanol	> 95%	40% (v/v)	100 ml
Acetic acid glacial	> 95%	10% (v/v)	25 ml
Distilled H <sub>2</sub> O			to 250 ml

### b. Sensitizing solution<sup>1</sup>

(30% ethanol, 0.125% glutardialdehyde, 0.2% sodium thiosulphate, 6.8% sodium acetate, dH<sub>2</sub>O)

	Stock concentration	Final concentration	Amount
Ethanol	> 95%	30% (v/v)	75 ml
Glutardialdehyde <sup>2</sup>	25% (w/v)	0.125% (v/v)	1.25 ml
Sodium thiosulphate	5% (w/v)	0.2%	10 ml
Sodium acetate (FW 82.03)	anhydrous	6.8%	17 g
Distilled H <sub>2</sub> O			to 250 ml

<sup>1</sup> Prepare always fresh.

<sup>2</sup> Add immediately before use.

### c. Silver solution<sup>1</sup>

(0.25% silver nitrate solution, 0.015% formaldehyde, dH<sub>2</sub>O)

	Stock concentration	Final concentration	Amount
Silver nitrate solution	2.5% (w/v)	0.25% (w/v)	25 ml
Formaldehyde <sup>2</sup>	37% (v/v)	0.015%	0.1 ml
Distilled H <sub>2</sub> O			to 250 ml

<sup>1</sup> Prepare always fresh in a dark container.

<sup>2</sup> Add immediately before use.

#### d. Developing solution<sup>1</sup>

(2.5% sodium carbonate, 0.0074% formaldehyde, dH<sub>2</sub>O)

	Stock concentration	Final concentration	Amount
Sodium carbonate (FW 105.99)	anhydrous	2.5% (w/v)	6.25 g
Formaldehyde <sup>2</sup>	37% (v/v)	0.00074%	0.05 ml
Distilled H <sub>2</sub> O			to 250 ml

<sup>1</sup> Stir vigorously to dissolve sodium carbonate.

<sup>2</sup> Add immediately before use.

#### e. Stop solution

(1.5% EDTA-Na<sub>2</sub>·H<sub>2</sub>O, dH<sub>2</sub>O)

	Stock concentration	Final concentration	Amount
EDTA-Na <sub>2</sub> ·H <sub>2</sub> O (FW 372.24)	anhydrous	1.5% (w/v)	3.65 g
Distilled H <sub>2</sub> O			to 250 ml

#### f. Gel preserving solution

(30% ethanol, 4% glycerol, dH<sub>2</sub>O)

	Stock concentration	Final concentration	Amount
Ethanol	> 95%	30% (v/v)	75 ml
Glycerol	> 87% (v/v)	4% (v/v)	11.5 ml
Distilled H <sub>2</sub> O			to 250 ml

#### g. Homogenization buffer (pH 7.4)

(250 mM sucrose, 3 mM imidazole, ddH<sub>2</sub>O)

	Final Concentration	Amount
Sucrose (FW 342.3)	250 mM	8.55 g
Imidazole (FW 68.08)	3 mM	0.021 g
Double distilled H <sub>2</sub> O		to 100 ml

### S. Sucrose solutions<sup>1</sup>

(Gram sucrose, 3 mM imidazole solution pH 7.4)

	Final Conc.	Amount	3 mM imidazole solution <sup>2</sup> pH 7.4
Sucrose (FW 342.3)	62%	31 g	to 50 ml
	35%	17.5 g	to 50 ml
	25%	12.5 g	to 50 ml
	10%	5 g	to 50 ml

<sup>1</sup> Different percentage of sucrose solution should be made separately.

<sup>2</sup> 3 mM imidazole solution must be prepared before in large amount.

### T. Sphingosine 1-phosphate (S1P) solution<sup>1</sup>

(0.001 g S1P, warm methanol)

	Stock concentration	Amount
Sphingosine 1-phosphate (MW- 379.5)	10 mM	0.001 g
Warm methanol (analytical grade)		to 0.26 ml

<sup>1</sup> Prepare in vacuum desiccators and aliquot aseptically as desired volume.

## 1.4 Mycobacterial species

*Mycobacterium tuberculosis* H37Rv, *Mycobacterium Smegmatis* mc<sup>2</sup>.

## 1.5 Human cells

Monocytes derived macrophages (MDM) from healthy volunteers, Human promonocytic THP-1 leukemia cell line.

## 1.6 Animal Model

Mouse Balb/C

## Methods

### 1. *In vitro* studies

#### 1.1 Mycobacterial culture and stock preparation

Pathogenic *M. tuberculosis* H37Rv (MTB) and nonpathogenic *M. smegmatis* mc<sup>2</sup> (Msm) strains were grown at 37°C/5%CO<sub>2</sub> incubator in Middlebrook 7H9 broth supplemented with 10% ADC. Mycobacteria after saturated growth were harvested, suspended with homogeneity in sterile PBS pH 7.2. Bacterial suspensions were sonicated for 3-5 minutes at low power output to disperse the bacteria without significant death of bacilli and aliquoted as 1ml stock, followed by freezing at -80°C until use. In case of Msm, 20% glycerol was added to each aliquot before freezing. Per milliliter colony forming units (CFU/ml) of bacilli were determined by plating the thawed bacilli on OADC supplemented 7H10 agar plates after appropriate dilution in PBS containing 0.015% Tween-80. Bacilli colony was counted at 3 days for Msm and at 21 days for MTB after incubation at 37°C/5%CO<sub>2</sub>. Bacillus population in each aliquot after CFU calculation found for Msm and MTB was 10<sup>9</sup>/ml and 1.5x10<sup>9</sup>/ml respectively. Each aliquots used for infection were also grown on 7H10 plates to re-confirm the CFU/ml of bacteria after thawing.

#### 1.2 Monocytes derived macrophages (MDM) isolation and differentiation

Peripheral blood mononuclear cells (PBMC) were isolated from healthy, purified protein derivative-negative human buffy coat blood preparations by centrifugation on ficoll-hypaque gradient and monocytes were separated by adherence as previously described [6]. In brief, the equal volume of room temperatured ficoll-hypaque was added on top of buffy coat. The total volume was brought to 50 ml by adding the RPMI 1640 and centrifuged for 20 minutes at 1800 rpm. Interface containing monocytes, lymphocytes and a few platelets were saved and washed thrice with RPMI 1640 by centrifugation each for 20 minutes at 1800 rpm, 1200 rpm and 800 rpm respectively. The pellet containing PBMC was dissolved in an appropriate volume of complete medium (see materials) and number of cells was counted after staining with trypan blue by using haemocytometer Neubauer chamber. Finally, PBMC cells were suspended at a concentration of  $6 \times 10^6$ /ml in complete medium. Monocytes were separated from lymphocytes by

adherence in tissue culture flask. To this aim,  $100 \times 10^6$  PBMC cells in 15 ml RPMI medium were transferred in polystyrene T75-cm<sup>2</sup> tissue culture flasks (Corning, Cambridge, MA) and were incubated for 1 hour at 37°C/5% CO<sub>2</sub>. After incubation, non-adherent cells (ie lymphocyte) were removed by 3 washes with warm RPMI 1640 and adherent cells were further incubated for overnight at 37°C/5%CO<sub>2</sub> after re-supplemented with complete medium. Next day, adherent monocytes were collected by gentle scraping with a cell scraper (Sarstedt, Newton, NC) after 15-minutes incubation with 5 mM EDTA in PBS at 4°C. Collected monocytes were finally centrifuged at 1800 rpm for 10 minutes. Pellets were re-suspended as  $1 \times 10^6$  cells /ml in complete medium and distributed in 24 well cell culture plates as  $1 \times 10^6$  cells /ml/ well, for 5-7 days to further differentiate into macrophages.

In each experiment, cells from a representative flask were analyzed via flow cytometry by CD14 staining and morphological parameters (forward scatter vs. side scatter) to assess monocyte purity, which was always >80% (data not shown).

### 1.3 THP-1 cells culture and differentiation

Human promonocytic THP-1 leukemia cell line, induced to differentiate, was also used in, *in vitro* experiments because of its similarity with alveolar macrophages [110]. In particular, the human promonocytic THP-1 leukemia cell line was maintained in culture in complete medium at 37°C/5%CO<sub>2</sub> in a humidified incubator. THP-1 cells grown in complete medium (see material) were collected and centrifuges at 1800 rpm / 10 minutes. Pellets were then dissolved in a small volume of complete medium and number of cells was counted after staining with trypan blue by using Neubauer chamber. Finally, the volume was adjusted by adding complete medium as to get  $1 \times 10^6$  cells /ml. Cells were then differentiated to macrophage-phenotype by stimulating with 20 ng/ml Phorbol 12-Myristate 13-Acetate (PMA) and distributed as  $1 \times 10^6$  cells /ml / well, in 24 well culture plates followed by incubation for 72 hours at 37°C.

Human MDMs or differentiated THP-1 (dTHP1) cells were then washed and reconstituted in complete medium, before use in experiments.

## 1.4 *In vitro* Infection and evaluation of mycobacterial growth

In order to evaluate the intracellular bacillary viability, human MDM and dTHP1 cells were infected for 3 hours at 37°C in 24 well plates, with MTB at a multiplicity of infection (MOI, bacteria/macrophage ratio) of 1 or with Msm at the MOI of 1 or 50, as indicated in the different colony forming unit (CFU) experiments. Bacterial inocula used for infection was plated immediately in 7H10 agar plates to determine the bacterial viable count as well as multiplicity of infection. Extra-cellular bacilli after 3 hours of infection were removed by washing the monolayer with RPMI 1640 twice, followed by re-suspension of macrophages monolayer in 1 ml complete medium.

To check the efficacy of S1P induced macrophages against MTB growth control, cells after removal of non-phagocytosed bacilli, were stimulated with either D-erythro-sphingosine-1-phosphate (S1P) or D-erythro-sphingosine (a synthetic, chemically manufactured product, by Calbiochem, San Diego, CA) at the concentration of 0.5, 5 and 50  $\mu$ M, and incubated at 37°C/5%CO<sub>2</sub> incubator for the times indicated in the different CFU experiments.

In order to assess the role of macrophage PLD activity in mycobacterial growth control, Msm infected MDM cells were further cultured for 1 and 24 hours with either 0.3% ethanol or 10 nM calphostin c (Sigma, St. Louis, Mo) which inhibit PLD activity by substituting for water as the nucleophilic acceptor of the phosphatidyl moiety [83] and by targeting PLD catalytic domain [121] respectively.

In order to assess the involvement of macrophage PLD activity in S1P-induced mycobacterial growth control, MTB-infected dTHP1 cells were co-cultured with 5  $\mu$ M S1P together with different concentration of either ethanol or 1- butanol for the different times as indicated in the figure 15. To this regard, short chain primary alcohols, inhibit PLD dependent PA generation and induce the formation of the metabolically inactive Pet-OH by substituting for water as the nucleophilic acceptor of the phosphatidyl moiety. Same concentration of 2-butoanl was also utilized as a control of transphosphatidylation reaction. CFU assay was performed as previously described [118]. Briefly, at immediately after 3 hours of infection and at the indicated time points after infection and stimulation in different experiments, supernatant of each well was removed and saved individually in a glass tube. Cells in the wells were lysed by adding 1 ml ice cold sterile PBS containing 0.1% Saponin (Sigma, St. Louis, MO) followed by incubation at 37°C for 30

minutes. Because prolonged *in vitro* culture of macrophages is accompanied by detachment of a minority of cells from the monolayer, viability of intracellular MTB was determined by combining adherent and suspension macrophages for each sample. Hence, sample from initial supernatant and lysed adherent fraction for each sample were combined, serially diluted in PBS containing 0.01% Tween-80, sonicated to avoid the mycobacterial clump and plated in triplicate on Middlebrook 7H10 agar. Msm and MTB colonies were enumerated after incubation of the plates at 37°C/5%CO<sub>2</sub> in humidified air for 3 and 21 days, respectively. Any modification, in terms of macrophage viability, was not detected in all experimental conditions used (data not shown).

### 1.5 Analysis of PLD activity

To analyze the role of PLD in intracellular mycobacterial growth control two sets of experiments were done. First, the role of PLD in natural growth control of pathogenic MTB H37Rv and non pathogenic environmental Msm was carried out, and second, the involvement of macrophage PLD in S1P induced antimycobacterial activity was pursued. Macrophage PLD activity was measured by the transphosphatidylase assay.

Briefly, 24 well plate-cultured dTHP1 and MDM cells were suspended in complete medium supplemented with 20 mM HEPES (pH 7.4). Cells were then labeled with 1 µCi/ml [<sup>3</sup>H]-Myristic Acid (Amersham, UK) for 180 minutes at 37°C, followed by washing with RPMI 1640 to remove unincorporated radioactivity. Thereafter, cells were incubated for 15 minutes at 37°C in complete medium with 1% ethanol to allow detection of [<sup>3</sup>H]-Pet-OH, as a specific transphosphatidylase reaction product of PLD.

To evaluate the differential modulation of PLD activity in course of pathogenic and non pathogenic mycobacterial infection, dTHP1 cells and MDM after radiolabelling and ethanol incubation, were infected with either MTB H37Rv or Msm at MOI of 1 for 0, 15, 180 minutes. At the indicated minutes post exposure, cells were washed twice with 20 mM HEPES (pH 7.4) and total lipids were extracted and separated by thin layer chromatography (TLC) in an ethyl acetate/isooctane/acetic acid/water (130/20/30/100 v/v) solvent system, as previously described [158]. Areas containing [<sup>3</sup>H]-Pet-OH were collected from silica gel plate as compared by standard Pet-OH and quantified by liquid scintillation spectrophotometer. The radioactivity present in Pet-OH fraction was expressed as the percentage of the total radioactivity collected from the lane.



To analyze the involvement of macrophage PLD in S1P induced anti-mycobacterial response, dTHP1 cells, after radiolabelling and ethanol incubation, were infected with MTB H37Rv at MOI of 1 for 3 hours at 37°C. Extra-cellular bacilli were removed by washing the monolayer with RPMI 1640 twice, followed by re-suspension of cells in 1 ml complete medium and stimulation or not with 5 µM S1P. At 15, 90, 180 minutes post-stimulation, cells were washed twice with 20 mM HEPES (pH 7.4) and total lipids were extracted and separated by TLC in an ethyl acetate/isooctane/acetic acid/water (130/20/30/100 v/v) solvent system, as above described. Areas containing [<sup>3</sup>H]-Pet-OH were collected from silica gel plate as compared by standard Pet-OH and quantified by liquid scintillation spectrophotometer. The radioactivity present in Pet-OH fraction was expressed as the percentage of the total radioactivity collected from the lane.

## 1.6 SDS-PAGE and Western blotting

Expression of PLD1, LAMP1, Cathepsin D was measured by using western blotting analysis as previously described [20]. In brief, dTHP1 cells, cultured in complete medium, were infected or not with MTB H37Rv at MOI of 1 for 3 hours at 37°C, washed twice and stimulated or not with 5 µM S1P at 37°C. At 24 hours post-stimulation, cells were suspended in lysis buffer [100 mM Tris/HCl (pH 7.4), 2 mM EDTA, 100 mM NaCl, 1% (v/v) Nonidet P40, 0.1% SDS, 2000 i.u./ml aprotinin, 1 mM PMSF, 100 µM leupeptin, 50 µM pepstatin A, 10 mg/ml IAA] and incubated in ice for 30 minutes. Lysate were centrifuged at 12000 rpm for 30 minutes at 4°C and supernatant containing protein were saved and stored in -20°C until use.

Amount of protein in each sample was determined by using MicroBCA™-Protein assay kit, commercially available from Pierce, UK, as per the recommended protocol. For each condition 10 µg total proteins were separated onto SDS-PAGE as per the protocol described by Laemeli *et al* [79]. According to the molecular weight of protein to be resolved, the percentage of SDS-PAGE gel was decided. In particular, LAMP 1 and PLD1 were resolved in 8% (w/v) SDS PAGE, whereas cathepsin D was in 12% (w/v) gel. Gels were prepared according to the recipe provided in the buffer and solutions appendix and total protein were separated with Tris-Glycine buffer system at 200 constant voltage utilizing Bio-Rad instruments. At the end of the run, proteins were transferred to the nitrocellulose membrane (NC) in a semidry system (Bio-Rad), at 15 constant volts for different times as per the molecular weight of protein of interest. Membrane were then dipped in PBS/T-20 (0.1% T-20 in PBS, pH 7.2) and washed extensively to remove

away any remnants of acrylamide of gel. The membranes were then blocked with blocking buffer (see buffer and solution) 2 hours at room temperature in an incubator shaker. Respective filters were then incubated overnight in a rotary incubator shaker at 4°C with mouse anti-human polyclonal -PLD (Quality Controlled Biochemicals, MA), mouse anti-human LAMP-1 and mouse anti-human cathepsin D (BD transduction laboratories) antibodies at 1:250, 1:1000, 1:1000 dilution respectively, diluted in blocking buffer. Next day, membranes were washed extensively 5 times with WB wash buffer, each with 5 minutes in shaker, followed by incubation for 2 hours at room temperature with Goat anti-mouse secondary antibodies conjugated with horse raddish peroxidase (HRP) (Bio-Rad), diluted 1:10000 in dilution buffer. After following further 5 washing as described above, membrane was incubated with HRP enzyme; "SuperSignal West Pico Chemiluminescent" (Pierce, UK) and signal intensity was captured for different time interval by exposing the membrane to the film "CL-Xposure" (Pierce, UK) as per the manufacturer recommended protocol.

## **1.7 Confocal fluorescent microscopy**

The degree of maturation of MTB-containing phagosome was assessed by analyzing the colocalization of bacilli with lysosomes after staining mycobacteria with auramine and lysosomes with the acidophilic dye LysoTracker Red (Molecular Probes, NL). dTHP1 monolayer, suspended in complete medium containing 20 mM HEPES (pH 7.4), was incubated with LysoTracker Red at a 1:10,000 dilution for 2 hours at 37°C. Unincorporated dye was removed and cells were infected for 3 hours with MTB. After removal of non-phagocytosed bacilli, LysoTracker Red was added again to each well for 30 minutes. Cells were washed and, incubated for 3 hours with 5 µM S1P in presence or absence of 0.3% ethanol. Thereafter, monolayer was washed with PBS, fixed by 15 minutes incubation with 3.75% paraformaldehyde at room temperature and permeabilized with ice-cold methanol-acetone (1:1) followed by further three washings with PBS. Cells were then seeded on poly-L-lysine (Sigma, MO) pretreated slides (VWR International Merck Euro lab, FR). The localization of MTB was determined by incubating infected monolayer with auramine (Becton Dickinson, MD) for 20 minutes at 25°C, followed by 3 minutes incubation in 0.5% acid alcohol and repeated washing with PBS. Finally, cover slips were mounted with Vectashield mounting medium H-1000 (Vector laboratories, CA) and edges were sealed with nail polish. Confocal fluorescence microscopy was performed on a Zeiss Laser Scan Inverted 510 microscope (Carl Zeiss Inc., Germany). An argon-krypton laser (excitation, 488 nm; emission band pass, 505-530 nm) was used for detection of auramine

fluorescence, and a helium-neon laser (excitation, 543 nm; emission limit of pass, 585 nm) was used for the detection of Lysotracker Red. Neither SIP nor ethanol directly affected the fluorescence of auramine or Lysotracker Red (data not shown).

## **2. *In vivo* studies**

### **2.1 Mice and experimental condition**

All the *in vivo* experiments and infection of mice with *M. smegmatis* (Msm) and *M. tuberculosis* (MTB) were conducted at center for Animal technology of University of Rome "Tor Vergata". BALB/c mice (female, 6 weeks old; Charles River laboratories, MA) were maintained in SPF condition (specific pathogen free) in a bio-safety level 3 facilities. Temperature (20±2°C) and humidity (55±5% relative humidity) were continuously monitored, whereas food (RF-18, Mucedola, Italy) and water were given *ad libitum*.

Mice utilized for Msm experiment were subdivided in 4 group's comprising 4 mice per group. 1<sup>st</sup> group received only Msm was called as positive infection control. Another 3 groups, received Msm and three different doses of SIP (1, 5, 20 nmoles). Whereas mice utilized for experiments conducted with MTB were divided in 6 different groups comprising 8 mice per group, except 1<sup>st</sup> group which had 12 mice which received only MTB and was called as positive infection control. These 12 mice were further subdivided into 4 and 8 mice per group as an early and late positive control respectively. Another 3 groups, received MTB and three different doses of SIP (1, 5, 20 nmoles). However, 5<sup>th</sup> group of mice known as negative control does not receive anything except physiological solution; while, last group of mice (i.e. 6<sup>th</sup> group) known as drug control, received 20 nmoles of SIP at every treatment schedule.

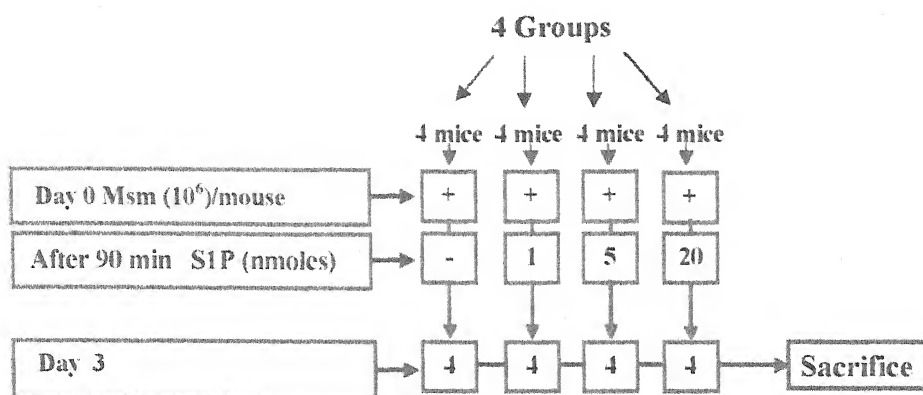


Figure 9. Group designing, treatment schedule and experimental scheme during *M. smegmatis* *in vivo* experiment.

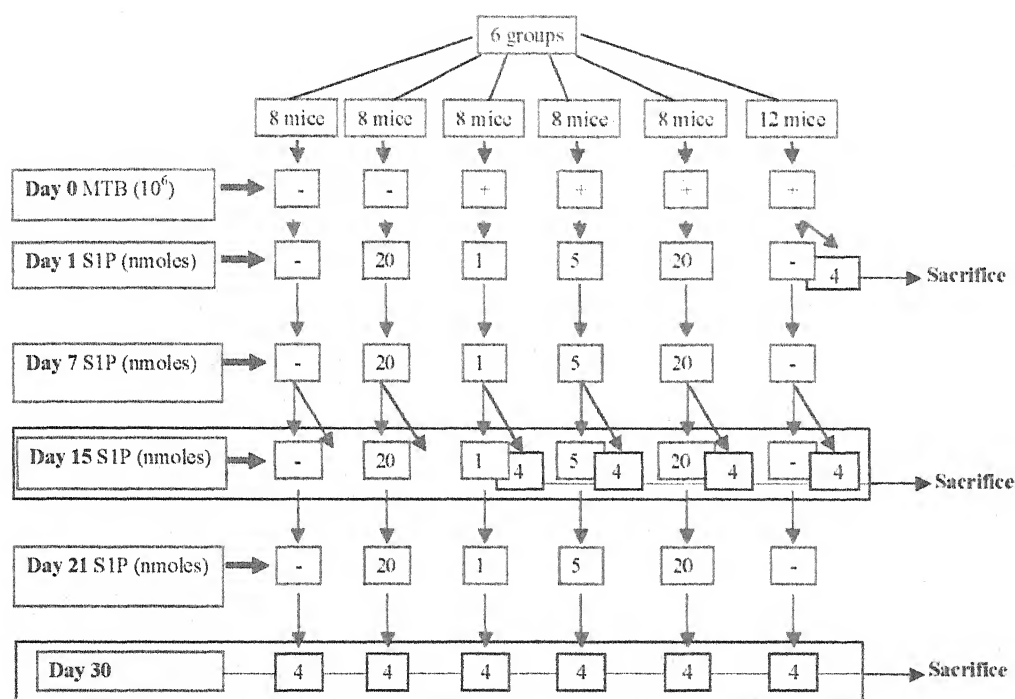


Figure 10. Group designing, treatment schedule and experimental scheme during *M. tuberculosis* *in vivo* experiment.

## 2.2 *In vivo* infection and CFU

Either mycobacteria used for infection were prepared as per the protocol described in *in vitro* experiments and were finally suspended at  $5 \times 10^6$  CFU/ml in sterile saline. Bacterial suspension was sonicated to avoid

the clumping and 100 µl of suspension containing 10<sup>6</sup> CFU of either Msm or MTB H37Rv was injected via the retro-orbital vein. Bacterial inoculum used was plated in 7H10 agar plates immediately after inoculation in order to determine the bacterial viable count. Msm-infected mice were received or not 1 nmoles, 5 nmoles, 200 nmoles of S1P (0.37 ng/ 100µl, 1.85 ng/ 100µl, 7.38 ng/ 100µl) through the same route at 90 minutes-, whereas MTB-infected mice were received or not the same doses of S1P at day 1, 7, 15, and 30- post-infection. Msm-infected mice were then sacrificed at day 3 post infection. MTB-infected mice were sacrificed at 14 and 30 days after infection, each comprising 4 mice respectively. Lungs and spleens from each mouse were collected aseptically, homogenized in a sterile saline and mycobacteria were enumerated by CFU assay as above described. From each mouse, lower lobe of the left lung was removed and used for histological analysis. The Animal Care and Use Committee of University of Rome "Tor Vergata" approved all experiments.

### 2.3 Histology analysis

Lung specimens from all four mice, each experimental group, were fixed in 10% buffered formalin, paraffin embedded, and 4 µm sections were stained with hematoxylin and eosin and examined for granuloma formation. In particular, to quantify the extension of the granulomatous reaction the entire surface of the lower lobe of left lung was analyzed. Moreover, areas of both granulomatous and healthy pulmonary parenchyma have been evaluated by Scion Image Analysis software (Scion Corporation, MA). The presence of MTB *in situ*, inside lung granulomas was evaluated by auramine stain (which binds with mycolic acid of mycobacterial cell wall) of "TB fluorescent stain kit M" according to the manufacturer's indications (Becton Dickinson, MD) and MTB were automatically quantified by Scion Image Analysis software by analyzing 20 granulomas per experimental group.

### 2.4 Preliminary Toxicology Studies

Negative control (physiological solution received) and drug control group (only S1P received) mice were administered once a week with 100 µl of sterile physiological solution and 20 nmoles of S1P respectively through retro-orbital vein for 6 weeks. Every week body weight of mice were taken and compared. Half

numbers of the mice in each group were also sacrificed, and lungs, spleen, and liver were isolated for histological studies as described above. Rest half left alive to check their normal viability.

### **3. *Ex vivo* studies**

#### **3.1 Isolation of alveolar macrophages from human bronco alveolar lavage (BAL) and *ex vivo* analysis**

In order to verify anti-mycobacterial efficacy of S1P in alveolar macrophages isolated from patients with pulmonary TB, *ex vivo* CFU experiment was carried out. In this regard, BAL from patients with pulmonary TB was collected from Division of Respiratory Disease, L. Spallanzani Hospital Rome. The patients data recruited in this study were documented and specimens collected from the patients admitted into hospital with pulmonary TB before they receive antibiotics treatment. To remove the mucous and other viscous impurities, BAL was initially filtered by using sterile 100  $\mu$ M filter. Filtrate was then centrifuges at 1300 rpm for 10 minutes at room temperature, followed by saving of supernatant separately for other experimental purpose. The pellets containing cells were washed thrice in cold PBS pH 7.2, followed by re-suspension in complete medium as to get  $1 \times 10^6$  cells /ml. Medium utilized to culture the cells was as of used for culturing of THP1 cells, except in place of Gentamycine (5  $\mu$ g/ ml), Ampicillin (5  $\mu$ g /ml) and fluconazole (2  $\mu$ g /ml) was added. Finally  $3 \times 10^6$  cells / 3 ml / well for each condition, was cultured in 6 well plates and stimulated or not with 5  $\mu$ M of S1P for 0 and 2 days. At indicated times points cells were lysed by adding 0.1% saponin directly into each well (Sigma, St. Louis, MO) followed by incubation at 37°C for 30 minutes. Lysate were collected, serially diluted in PBS containing 0.01% Tween-80 (Merck, Germany), sonicated to avoid the mycobacterial clump and plated in triplicate on Middle brook 7H10 agar (Becton Dickinson, MD). MTB colonies were enumerated after incubation of the plates at 37°C/5%CO<sub>2</sub> in humidified air for 21 days.

### **4. Statistics**

Comparison between groups were done using Student's t test as appropriate for normally distributed data. Mann-Whitney test was performed for data that were not normally distributed.  $p < 0.05$  was considered statistically significant.

## 5. Proteomic analysis

### 5.1 Two-dimensional electrophoresis (2-DE)

#### 5.1.1 Infection and Sample preparation

Identification of S1P induced macrophage novel molecules involved in mycobacterial killing was pursued using two-dimensional gel electrophoresis. In brief, dTHP1 cells, cultured in complete medium, were infected or not with MTB H37Rv at MOI of 1 for 3 hours at 37°C, washed twice and stimulated or not with 5 µM S1P at 37°C. At 24 hours post-stimulation, media from each well were replaced with 1 ml cold PBS pH7.2, and plates were incubated in ice for at least 1 hour. Thereafter, cells were collected and centrifuged at 1600 rpm for 10 minutes at 4°C. Pellets were further washed with cold PBS and finally lysed in ice using SDS lysis buffer (see buffer and solution) containing protease inhibitors. Microcentrifuge tube containing lysate were placed in 90°C water bath for 15 minutes followed by centrifugation at 12000 rpm / 4°C for 30 minutes. Supernatant containing protein were collected and stored at -20°C until further use.

#### 5.1.2 Protein quantification

Amount of protein in each sample was determined by using MicroBCA™-Protein assay kit, commercially available from Pierce, UK, as per the recommended protocol. In brief, MicroBCA™-Protein assay reagent kit is a detergent-compatible bicinchoninic acid formulation which is a highly sensitive reagent for the quantitative colorimetric determination of total protein in dilute aqueous solution. This unique reagent system utilizes bicinchoninic acid (BCA) as the detection reagent for  $\text{Cu}^{++}$ , which is formed when  $\text{Cu}^{++}$  is reduced by protein in an alkaline environment [127]. The purple-colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion ( $\text{Cu}^+$ ). This water-soluble complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentrations. The macromolecular structure of protein, the number of peptide bonds that the presences of four amino acids (cysteine, cystine, tryptophan and tyrosine) are reported to be responsible for color formation with BCA. Below written protocol is a little modified form of manufacturer recommended one, which is a protocol for microwell plate with a linear working range of 1-20 mg/ml.

Serially dilute the BSA standard provided with the kit in ddH<sub>2</sub>O and pipette the 100 µl of each standard or unknown sample (protein to be used for 2-DE) in duplicate into the appropriate microwell plate well. 100µl of the diluent (ddH<sub>2</sub>O for BSA standard and SDS lysis buffer for protein sample to be used for 2-DE analysis) was used for the blank wells. Working reagent was prepared by mixing the MicroBCA™ reagent -MA, -MB, -MC in a ratio of 25:24:1 and 100 µl was added into each well containing 100 µl of protein sample to be analysed. Mixed and covered the plate and incubated at 37C for 2 hours. After incubation, cooled the plate to room temperature and measured the absorbance at or near 562 nm on a plate reader. the average 562 nm reading for the blanks was subtracted from the 562 nm reading for each standard or unknown sample. A standard curve was prepared by plotting the average blank corrected 562 nm reading for each BSA standard vs. its concentration in µg/100 µl. Using the standard curve, protein concentration for the unknown sample was determined.

### 5.1.3 Sample purification

In order to achieve a well-focused first dimension separation, sample protein must be free of non-protein impurities such as salts, residual buffers, ionic detergents, nucleic acids, and other charges small molecules that carry over from sample preparation. Hence treatment of protein sample with PlusOne 2-D Clean-Up Kit was carried out to improve the quality of 2-D electrophoresis results, resulting in reduced - streaking, -background staining and -other consequences of interfering contaminants.

1-100 µl of protein sample (containing 1 µg to 1 mg protein) were transferred into 1.5 ml microcentrifuge tube and followed the all steps with the tubes in an ice bucket unless otherwise specified. 300 µl precipitant (provided) was added to the 1-100 ul of protein sample and mixed well by vortexing or inversion followed by incubation on ice for 15 minutes. To this 300 µl co-precipitant (provided) was added and mixed by vortexing briefly. Tubes were centrifuged at 12000 rpm for 5 minutes followed by removal of maximum possible supernatant by decanting or careful pipetting. Thereafter, carefully repositioned the tube in centrifuge as before and centrifuge for 30 seconds (a brief pulse) to remove any visible liquid. Pipetted 50 µl of co-precipitant on the top of each pellet and centrifuged at 12000 rpm for 5 minutes. Supernatant was discarded and 25 µl of ddH<sub>2</sub>O was pipetted on the top of each pellet and vortexed until the pellet is not dispersed. To this 1 ml of wash buffer (provided with kit which has been pre-chilled for at least 1 hour at -20°C) and 5 µl wash additive was added followed by vortex ting until the pellet is fully dispersed, but will not dissolve in wash buffer. Tubes were then incubated at -20°C for at



least 30 minutes followed by vortexing for 20-30 seconds once every 10 minutes. Tubes were then centrifuged for 5 minutes for at least 12000 rpm at 4°C followed by carefully remove and discard the supernatant. A visible white pellet was then allowed to air dry briefly for no more than 5 minutes. Re-suspended the each pellet directly in appropriate volume (375 µl for 18 cm IPG strips) of rehydration solution with IPG buffer (see buffer and solution) for first dimension IEF. Vortexed the tubes to dissolve the pellets completely and centrifuged at 12000 rpm for 5 min at 15°C to remove any insoluble material and to remove any foam. The supernatant was loaded directly onto first dimension IEF or may be transferred to another tube and stores at -80°C for later analysis.

#### **5.1.4 IPGphor Isoelectric focusing**

First dimensional isoelectrofocussing was performed with precast IPG strips (pH 3-10NL, 180x3x0.5mm, Amersham Biosciences) on IPGphor Isoelectric focusing system (Amersham pharmacia biotech), which allows the rehydration of the IPG strip and IEF in individual strip holders. A strip holder is made up of thermally conductive ceramic with built-in platinum electrodes and a transparent lid. Different-length strip holders are available for the different-length IPG strips.

Protein samples prepared above (375 µl) were pipetted into each strip holder by delivering the solution slowly at a central point in the strip holder channel away from the sample application wells. Protective cover of the IPG strip was removed and IPG strips were positioned with the gel side down and pointed (anodic) end of the strip directed toward the pointed end of the strip holder. Even and complete wetting of strips and absence of large bubbles (if any) between solution and strips, and contact of strips with in-built electrodes were ensured. In order to minimize evaporation and urea crystallization IPG cover fluid were applied on the top of strip until the entire IPG strips was covered. Thereafter, put the lid on strip holder to ensure that the IPG strips maintains good contact with the electrodes as the gel swells. Put all the above assembly on the IPGphor unit platform and safety lid of instruments were closed. Strips were then allowed to rehydrated for 16 hours followed by application of 33950 volt-hours in 5 different steps : (100 Vh, 2 hours; 500 Vh, 1 hour; 1000 Vh, 1 hour; 8000 Vh, 4 hours; 250 Vh, 1 hours; at 50 mA current / IPG strips and at 20°C constant temperature ). Moreover, according to the length of IPG strips used, below given Amersham Biosciences recommended program table (Table 5) can also be selected. It is generally preferable to program a protocol on the basis of volt-hours rather than time.

**Table 5. Immobiline drystrip IEF guidelines for IPGphor Isoelectric focusing system.**

**50 mA per IPG strip**

**20°C for both rehydration and IEF**

**For pH gradients 4-7, 3-10L, and 3-10NL**

	Step	Voltage	Step duration (hr:min)	volt-hours (Vh)	Gradient type
7 cm		rehydration	12:00 <sup>1</sup>		
	1	500	0:30	250	Step-n-hold
	2	1000	0:30	500	Step-n-hold
	3	8000 <sup>2</sup>	1:00	8000	Step-n-hold
	4 <sup>3</sup>	250	0:30	125	Step-n-hold
11 cm		rehydration	12:00 <sup>1</sup>		
	1	500	1:00	500	Step-n-hold
	2	1000	1:00	1000	Step-n-hold
	3	8000 <sup>2</sup>	2:00	16000	Step-n-hold
	4 <sup>3</sup>	250	0:30	125	Step-n-hold
13 cm		rehydration	13:00 <sup>1</sup>		
	1	500	1:00	500	Step-n-hold
	2	1000	1:00	1000	Step-n-hold
	3	8000 <sup>2</sup>	2:00	16000	Step-n-hold
	4 <sup>3</sup>	250	1:00	250	Step-n-hold
18 cm		rehydration	14:00 <sup>1</sup>		
	1	500	1:00	500	Step-n-hold
	2	1000	1:00	1000	Step-n-hold
	3	8000 <sup>2</sup>	4:00	32000	Step-n-hold
	4 <sup>3</sup>	250	1:00	250	Step-n-hold

<sup>1</sup>The total rehydration time is required to be adjusted somewhat for convenience, but found must to be greater than 12 hours

<sup>2</sup>This voltage does not reached within the written step duration

<sup>3</sup>Step 4 is optional, which reduces the tailing effect of protein spot. For small strip length it may not be very useful but found to be good for higher length.

### 5.1.5 Second-dimension SDS-PAGE

After IEF, the strips were removed and either stored in  $-80^{\circ}\text{C}$  for further use or directly prepared for the second-dimensional SDS-PAGE, which was performed on Protein<sup>®</sup> II Xi cell (Bio-rad). For SDS-PAGE, 12% single percentage gels with 1.5 mm thick spacer were selected as it offers better resolution. Formulation of the gel solution was calculated and recipe was mixed for the required percentage of gel as given in table (Table 6) below.

**Table 6. Recipes for single percentage gels**

**(Preparation of stock solution is described in the appendix buffer and solutions of material sections)**

Final gel Concentration	5%	7.5%	10%	12%	15%
Monomer stock Solution (Acrylamide:bis; 30:0.8)	16.7 ml	25 ml	33.3 ml	40 ml	50 ml
Double distilled water	56.8 ml	48.5 ml	40.2 ml	33.5 ml	23.5 ml
4x resolving gel buffer (pH 8.8)	25 ml	25 ml	25 ml	25 ml	25 ml
10% SDS	1 ml	1 ml	1 ml	1 ml	1 ml
10% Ammonium per sulphate*	500 $\mu\text{l}$	500 $\mu\text{l}$	500 $\mu\text{l}$	500 $\mu\text{l}$	500 $\mu\text{l}$
TEMED*	50 $\mu\text{l}$	50 $\mu\text{l}$	50 $\mu\text{l}$	50 $\mu\text{l}$	50 $\mu\text{l}$
Total Volume	100 ml	100 ml	100 ml	100 ml	100 ml

\*add after deaeration

Gel solution was prepared in a vacuum flask, omitting the TEMED and APS and residual air was removed by apply a vacuum for a several minutes while stirring on a magnetic stirrer. APS and TEMED were added and mixed by gently swirling the flask, being careful not to generate bubbles and immediately poured the gel in gel cassette assembly until 3 to 10 mm below the top. In order to minimize gel exposure to oxygen and to create a flat gel surface, immediately after pouring, overlaid each gel with a thin layer of ddH<sub>2</sub>O and allowed a minimum of 1 hour for polymerization at room temperature. Water over the gel surface was replaced with gel storage solution followed by overnight polymerization at  $4^{\circ}\text{C}$ .

IPG strip were equilibrated in the SDS equilibration buffer containing 10 mg DTT / ml of buffer and incubated in the rotary shaker for 15 minutes. Replaced the SDS equilibration buffer with another equilibration buffer containing 25 mg/ ml of iodoactamide and incubated for other 15 minutes. Finally discarded the buffer and dip the IPG strip in the SDS electrophoresis buffer to lubricate it. IPG strips were positioned between the plates on the surface of the second dimension gel with the plastic backing against

one of the glass plates and with the help of thin plastic ruler IPG strips were pushed down until the close contact with top surface of slab gel. Thereafter, 0.5% melted agarose sealing buffer (40-50°C) was poured on the imbedding IPG strips, which prevents it from moving or floating in the electrophoresis buffer. Finally whole assembly was put in to electrophoretic chamber containing SDS electrophoresis buffer and electrophoreses was performed in 4°C cold room at constant current in two steps, while circulating the outer buffer with a magnetic stirrer. During the initial migration and stacking period, the current was approximately half of the value required for the separation.

Electrophoresis was stopped when the dye front was approximately 1 mm from the bottom of the gel. Gels were removed from the casting assembly and were stained using appropriated staining method.

### 5.1.6 Silver Staining

Silver staining was carried out using “silver staining kit for protein” (available from Amersham Pharmacia Biotech), which is a reagent kit for the fast, easy, reproducible and sensitive staining of protein in polyacrylamide gels. The high sensitivity of the visualization technique allows detection of most proteins down to the nanogram range, which is 100 times more sensitive than Coomassie Brilliant Blue.

Complete staining procedure was performed with gentle shaking of staining tray until unless stated and in order to avoid finger print contamination, in all steps gloves were used to handle the gel. In the first step, after the completion of SDS-PAGE, gels were soaked in fixing solution for 30 minutes, till the dye front stain turn green. Fixing solution were then replaced with sensitizing solution and left shaking for at least 30 minutes. Gels were then washed three times for a minutes each time followed by incubation with silver nitrate solution for 20 minutes in dark. Gels were then rinsed twice in ddH<sub>2</sub>O for one minute each time. Developing solution was added with shaking till the desired staining is reached. Reaction was terminated by incubating the developed gel in stop solution for 10 minutes followed by preserving the gel in preserving solution.

A Mass Spectrometry compatible silver staining protocol was also utilized in order to excise and analyze the protein spots from gels by Matrix Assisted Laser Desorption Ionization (MALDI)-Time of Flight (TOF). All the steps were same as written above for a standard silver staining protocol except the solution concentration and in some cases composition utilized. In brief, gels soaked in fixing solution (CH<sub>3</sub>OH:CH<sub>3</sub>COOH:ddH<sub>2</sub>O, 45:5:45) for 20-30 minutes. Gels were then rinsed with ddH<sub>2</sub>O for 30-60 minutes to remove the acid. Gels were then incubated for 1-2 minutes with sensitizing solution containing

0.02%  $\text{Na}_2\text{S}_2\text{O}_3$  in  $\text{ddH}_2\text{O}$ . Gels were then washed three times for a minute each time followed by incubation with 0.1% silver nitrate solution for 30 minutes in dark. Gels were then rinsed twice in  $\text{ddH}_2\text{O}$  for one minute each time. Developing solution (0.04%  $\text{HCHO}$ , 2%  $\text{Na}_2\text{CO}_3$  in  $\text{ddH}_2\text{O}$ ) was added with while shaking the gel. Solution was replaced if it turned yellow during developing process. Reaction was terminated when desired staining was obtained by incubating the developed gel in stop solution (1% acetic acid) for 10 minutes followed by preserving the gel in 1% acetic acid at  $4^\circ\text{C}$ .

### 5.1.7 Image Analysis

Following 2-DE and protein staining, the gels were digitized with Silver Fast scan software (Epson, CA, USA) on an Epson Expression 800 scanner (model G710U). The maximum resolution was kept was 12 800 x 12 800 dots per inch (dpi), and the same scanning parameters were used for all 2-D gels in a match set (reflective, positive, 36-bit color, 200dpi, same out put size, RGB (Red-Green-Blue) scan). The scanned data were presented in a .tiff file, and were acquired to PDQuest<sup>®</sup> image analysis software version 6.0 (Bio-Rad) for proteins spots analysis. PDQuest<sup>®</sup> image analysis software provides the essential tools for analyzing complex protein samples separated by 2-D electrophoresis. The software accurately landmarks proteins for gel alignment and identifies up or down-regulation of proteins based on intensity of protein staining. Following the acquisition of scanned gel image in to software, image were cropped and oriented, spots were detected, identified, compared and matched, data were normalized, analyzed and then report were prepared. The procedure of spot detection and identification includes the following steps. When the spots were appeared in PDQuest<sup>®</sup>, the original image was filtered and smoothed to clarify the spots, and three-dimensional Gaussian spots were created from the clarified spots. Three separate images were created: the original unaltered scan (2-D scan), the filtered and processed scan (filtered scan), and the synthetic images that contained the Gaussian spots (Gaussian scan image) with a defined volume and quality. All subsequent spot matching and analysis steps in the PDQuest<sup>®</sup> software were performed on Gaussian spots. In order to accurately perform a comparison between 4 different experimental conditions, same spot detection parameters were set as follows: sensitivity: 13.78; operator size: 9; mean peak: 3456; background floating ball radius: 43; background smoothing power mean kernel size 3x3. Proteins spots were automatically detected, background were corrected, spot density were quantified, and spots were matched between up to 8 gels. Images were checked by eye for undetected or incorrectly detected spots. The total staining intensity within each detected spot, that is the amount of protein present, is defined as spot volume. To compare spot volumes across different gels, the protein spots detected in each

experimental gel, i.e. the 'slave gel', were matched to the corresponding protein spots within a digitized reference spot pattern obtained from a selected experimental gel, i.e. the 'master gel'. To increase the likelihood that the highest possible number of spots in each slave gel could be matched to the corresponding spot in the master gel, selection of the master gel was based on the following criteria: high number of detected spots; best spot resolution across the whole proteome and the least horizontal striking (horizontal lines across the proteome due to partial precipitation of certain proteins). Automatic matching of spots in each slave gel to corresponding spots in the master gel was performed by selecting 1 or 2 landmarks across all gel, resulting in automatic matching of all spots. Slave spots that were incorrectly matched to master spots, after automatic matching, were corrected by removal of incorrect matching landmark and manual insertion of a new matching landmark. In order to correct for background staining, background intensity was subtracted from the spot volume values. To correct for differences in staining intensity between gels, spot volumes were divided by the volume of all spots and multiplied by the summarized total area of all spots. The resulting values represent the 'normalized spot volumes'.

## **5.2 Mass spectrometry**

### **5.2.1 In-gel trypsin digestion**

Protein/ peptides to be analyzed by Mass Spectrometry were excised from gels and were processed, digested by following the in-gel digestion protocol.

Gels containing the protein spots were rinsed thoroughly with ddH<sub>2</sub>O and bands of interest were excised using clean scalpel. All the protein spots were processed carefully separately in order to avoid cross contamination. A chopped spot was transferred into a micro-centrifuge tube and was washed again with 100-150 µl of ddH<sub>2</sub>O followed by centrifugation and discard of supernatant liquid. Gel spot was shrunken by adding acetonitrile (3-4 times equal the volume of gel spot) for 10-15 minutes and then acetonitrile was discarded by spinning down the gel particles in a vacuum centrifuge. At this stage the gel spot became white and stacked together. Gels particles were then swelled by adding 10mM DTT/0.1M NH<sub>4</sub>CO<sub>3</sub> followed by incubation at 56°C for 30 minutes to reduce the protein. Spin downed the gel particles and liquid was discarded followed by re-shrunked the gel particles by incubating with acetonitrile. Acetonitrile was replaced with 55mM IAA/0.1M NH<sub>4</sub>CO<sub>3</sub> and incubated for 30 minutes at room temperature in the dark. IAA solution was removed and gel particles were washed with 150-200 µl of 0.1M NH<sub>4</sub>CO<sub>3</sub> for 15

minutes. Resulting gel pieces were again shrunken by incubating with acetonitrile and spined down in a vacuum centrifuge. Supernatant was discarded and gel pieces were dried completely in a vacuum centrifuge. Gels particles were then re-hydrated in the digestion buffer containing 50 mM  $\text{NH}_4\text{CO}_3$ , 5mM  $\text{CaCl}_2$  and 12.5 ng/ $\mu\text{l}$  of trypsin at 4°C (ice bucket) for 30-45 minutes. At this step, after 20 minutes of incubation, check out the sample and add more buffer if all liquid is absorbed by the gel pieces. There after supernatant was discarded the gel pieces were incubated over night with 5-25  $\mu\text{l}$  of same buffer but without trypsin.

### 5.2.2 Extraction of tryptic peptides

Following morning spin down the tubes at 4000 g and saved the supernatant (called as A) in a clean autoclaved eppendorf. 20  $\mu\text{l}$  1% TFA was added to the each gel pellet and incubated for 20 minutes at room temperature followed by vortexing for 1 minute in every 5 minutes. Tubes were then centrifuges at 4000 g for 5 minutes and supernatant (B) were mixed with supernatant A. Thereafter gel pellets were incubated for 20 minutes with 25  $\mu\text{l}$  of acetonitrile:TFA (1:1) 0.1%, and were vortexed for 1 minutes in every 5 minutes. Supernatant (C) were saved after centrifugation at 4000 g for 5 minutes and mixed with supernatant A+B. Supernatant (A+B+C) was partially dried in a vacuum centrifuge (upto a volume of 3-5 $\mu\text{l}$ ) and then again reconstituted with 20  $\mu\text{l}$  of 0.1% TFA followed by vacuum centrifugation until the liquid volume reached upto 2  $\mu\text{l}$ . This 1  $\mu\text{l}$  of tryptic extract (supernatant A+B+C) was mixed with 1  $\mu\text{l}$  of  $\alpha$ -cyano-4-hydroxycinnamic acid (HCCA) in order to prevent the sample crytalization. Finally, 1  $\mu\text{l}$  the above mixture was directly spotted onto the stainless stell target of the MALDI instruments, according to the dried drop method. The sample was allowed to crystallize for 1 hour at room temperature and were analysed on MALDI-TOF MS.

### 5.2.3 MALDI-TOF MS

MALDI-TOF was used to obtain the PMF (peptide mass fingerprinting) data for each protein. All analysis was performed on Autoflex<sup>®</sup> mass spectrometer (Bremen, Germany). MALDI-TOF mass spectra were aquired with a pulsed nitrogen laser (337 nm) in reflector mode. In positive reflector mode spectra were obtained with these following instruments parameter: a pulsed ion extraction time of 19 kV,

accelerating voltage 20 kV, grid voltage 57%, mirror voltage ration 1.08, guide wire 0 is 0.07%, detector gain voltage of 1400 V and a laser frequency of 5 Hz. The acquisition range was between  $m/z$  500-3500, and low mass gate 300Da. The final mass spectrum was produced by averagining 500 laser shots. A protonated molecule ion,  $[M + H]^+$ , is generally produced by MALDI-TOF MS. The mass spectra were processed with the Bruker's data-processing software (flex analysis) in order to obtain the accurate monoisotopic peaks. Toward that end, the mass spectrometer was usually mass calibrated with a standard mixture that contained peptides with known molecular weight (angiotensin I, II, substance P and bombesin). Monoisotopic peaks obtaining after analysis was the PMF data that were used to search database.

### 5.2.4 Database analysis

The MALDI-TOF MS PMF data were used to identify each protein by a search on the SWISS-PROT database with PeptIdent software (<http://expasy.org/tool/peptident.html>). The parameters for the database search with PMF data are summarized in the **table 7**. The criteria to evaluate the data base results are summarized in the **table 8**.

**Table 7. Parameters for the databse search with PeptIdent software, based on PMF data**

- |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <ul style="list-style-type: none"> <li>a. Remove all peaks from trypsin, matrix, keratins and other unknown contaminants;</li> <li>b. SWISS-PROT databae;</li> <li>c. Search within the experimental <math>pI</math> value <math>\pm 1.00</math>, and experimental <math>MW \pm 20\%</math> (up to 50-100% if the protein is a suspected glycoprotein);</li> <li>d. <i>Homo sapiens</i> (human) species;</li> <li>e. <math>[M + H]^+</math> and monoisotopic peaks were selected;</li> <li>f. Modifications include cysteins treated with iodoacetamide (IAA), and methionines are oxidized;</li> <li>g. Trypsin, with one allowed missed cleavage site;</li> <li>h. Mass-tolerance unit = ppm.</li> </ul> |
|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|



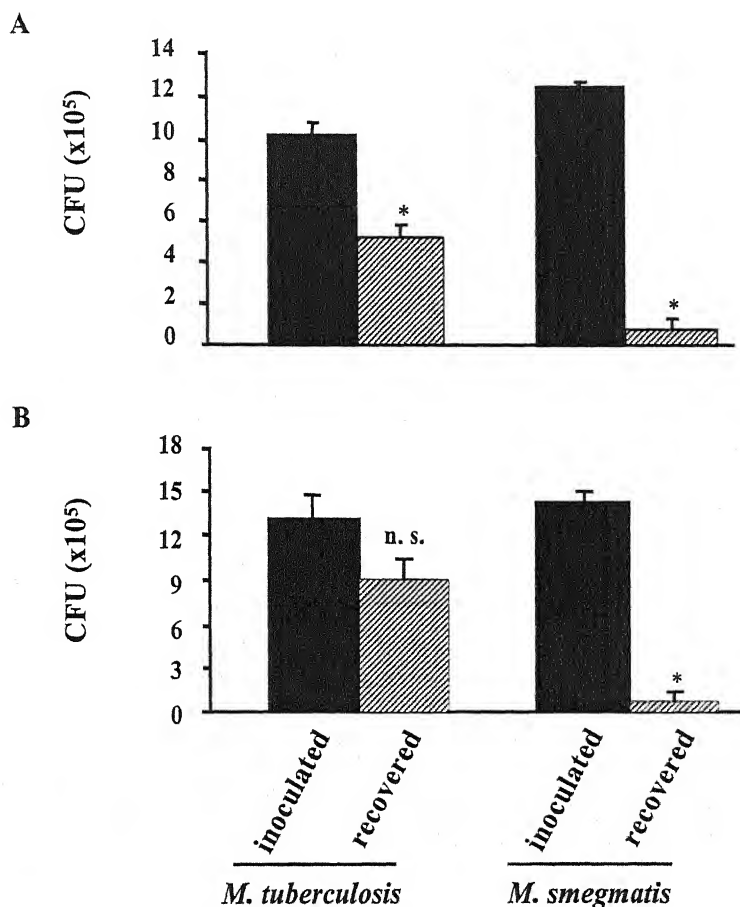
**Table 8. Criteria to evaluate the database search results with PMF data**

- a. Number of matched criteria peptides  $\geq 4$ ;
- b. The correct protein should be the top protein;
- c. (The number of matched-peptides of the top protein) – ( the number of matched-peptides of the second protein))  $\geq 2$ ;
- d. The matched-peptides should posses a higher signal intensity in the PMF data;
- e. Sequence coverage  $\geq 15\%$  (if the protein is very big, then the sequence coverage might be  $> 15\%$ );
- f. The top protein must appear when the mass tolerance was  $\leq 2000$  ppm;

## Results

### *M. tuberculosis* and *M. smegmatis* are differently controlled by human macrophages

To analyze the antimicrobial activity exerted by macrophages against MTB or Msm, both differentiated THP-1 (dTHP1) cells and monocytes-derived macrophages (MDM) were infected with either MTB H37Rv or *M. smegmatis* mc<sup>2</sup> at 1 bacillus per cell for 3 hours as described in material and methods.



**Figure 11. *MTB* and *Msm* are differently controlled by human macrophages.**

dTHP1 cells (panel A) and MDM (panel B) were exposed for 3 hours to MTB and Msm (MOI of 1). CFU assay was performed on the initial used inoculums and on infected cells to evaluate intracellular mycobacterial viability after 3 hours of phagocytosis. CFU results are shown as means + S.D. of triplicate values. dTHP1 cell data are representative of three different experiment (panel A), whereas MDM data are

representative of three individual experiment performed on macrophages derived from separate donors (panel B). Panel A. Bacteria recovered from MTB-infected dTHP1 in comparison to inoculated:  $p=0.001$ ; bacteria recovered from Msm-infected dTHP1 in comparison to inoculated:  $p=0.0007$ ; bacteria recovered from Msm-infected dTHP1 in comparison to MTB-infected dTHP1:  $p=0.005$ . Panel B. Bacteria recovered from MTB-infected MDM in comparison to inoculated:  $p=n.s.$ ; bacteria recovered from Msm-infected MDM in comparison to inoculated:  $p=0.0004$ .

Intracellular colony-forming units (CFU) were enumerated and compared to the initial infected inoculums. To exclude any interference due to the different mycobacterial replication rate, samples for CFU assay were collected at 3 hours after mycobacterial exposure. As depicted in **figure 11**, a mean 70.5% and 50% of the original MTB inoculum *versus* 0.8% and 0.25% of the original *M. smegmatis* inoculum were recovered after infection in dTHP1 cells (panel A) and in MDM (panel B) respectively, showing that human macrophages are able to strongly reduce the viability of non-pathogenic Msm but not that of the pathogenic MTB.

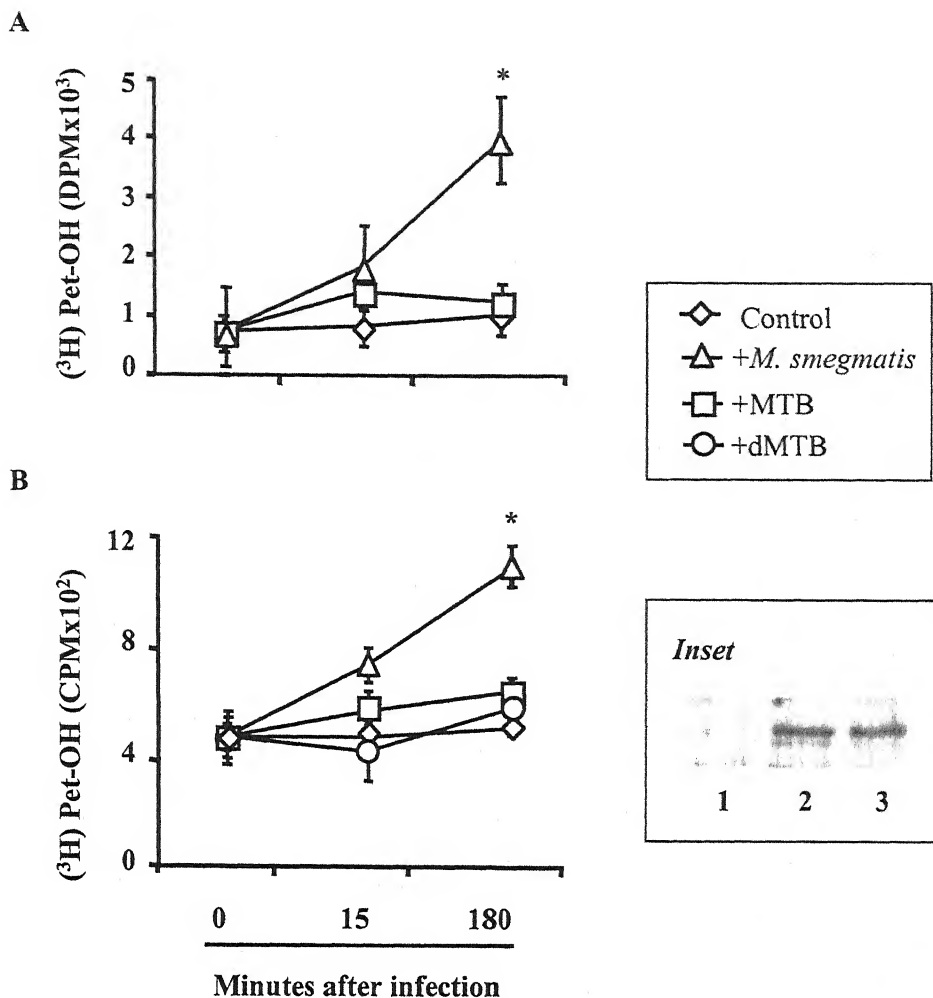
Moreover, in order to check whether less Msm recovery is dependent on less bacterial uptake, the rate of phagocytosis of both mycobacteria after infection was performed. For this purpose, supernatants of 3 hours infected macrophages were collected separately, and CFU assay were performed. Results indicate that 90-95% of both mycobacteria were phagocytosed both under control conditions, as well as in experiments where other chemicals were used before infection. (data not shown).

### ***Infection of human macrophages with pathogenic MTB or non pathogenic Msm results in a different modulation of PLD activity***

PLD has been recently shown to be involved in ATP-induced killing of intracellular mycobacteria by human macrophages [41]. Since macrophages show a differential ability to control intracellular replication of pathogenic and non-pathogenic mycobacteria such as MTB and Msm, we tested the hypothesis that such difference in intracellular growth control could reflect differences in host PLD activity. Thus, human macrophage PLD activity was quantified in response to either pathogenic or non-pathogenic mycobacteria by monitoring the accumulation of phosphatidyl ethanol ( $[^3\text{H}]\text{-Pet-OH}$ ) in infected dTHP1 or MDM. **Figure 12 A** shows the formation of  $[^3\text{H}]\text{-Pet-OH}$  in dTHP1 cells at immediately after exposure (time 0), and after 15 minutes and 3 hours of exposure to either MTB or Msm at the MOI of 1. In particular, MTB-infected dTHP1 showed no significant differences with uninfected cells, whereas Msm-

infected dTHP1 maintained a significant PLD activity profile throughout the infection, reaching the maximum of activation at 3 hours. At 15 minutes however, MTB infected THP-1 cells also showed higher but non significant PLD activity. This may be due to involvement of membranous PLD in the phagocytosis. As two different kind of PLD has been suggested; Membranous basically involved in phagocytosis and cytoskeleton organization and does not require  $\text{Ca}^{++}$  for their activity, whereas other cytoplasmic PLD suggested to anti-microbial and require  $\text{Ca}^{++}$ . The same result was obtained repeating the same experiment in MDM at 0, 15, 3 hours after infection (**Figure 12 B**). Interestingly, in order to see the whether avoidance of PLD activation by MTB is a viable phenomenon, PLD activity was analyzed also after infection with heat killed MTB. Heat-killed MTB did not induce significant higher PLD activity than those in live-MTB at 15 (live: 454+45 [3H]-Pet-OH cpm; dead: 328+67 cpm; p=n.s.) and 180 minutes (live: 470+28 [3H]-Pet-OH cpm; dead: 432+24 cpm; p=n.s.) after infection (**Figure 12 B**).

To see whether differences in PLD activity could reflect differences in protein expression, macrophage PLD1 expression was analyzed in MDM at 24 hours after infection with either mycobacterial strains by western blotting analysis. Results reported in the **inset of figure 12 B** shows that higher levels of PLD1 expression were observed in both MTB- and Msm-infected MDM when compared to uninfected MDM cells. However, no significant differences in the PLD1 expression levels were revealed while comparing between either mycobacterial strains infected MDM.



**Figure 12. Infection of human macrophages with pathogenic MTB or non pathogenic Msm results in a different modulation of PLD activity but not expression.**

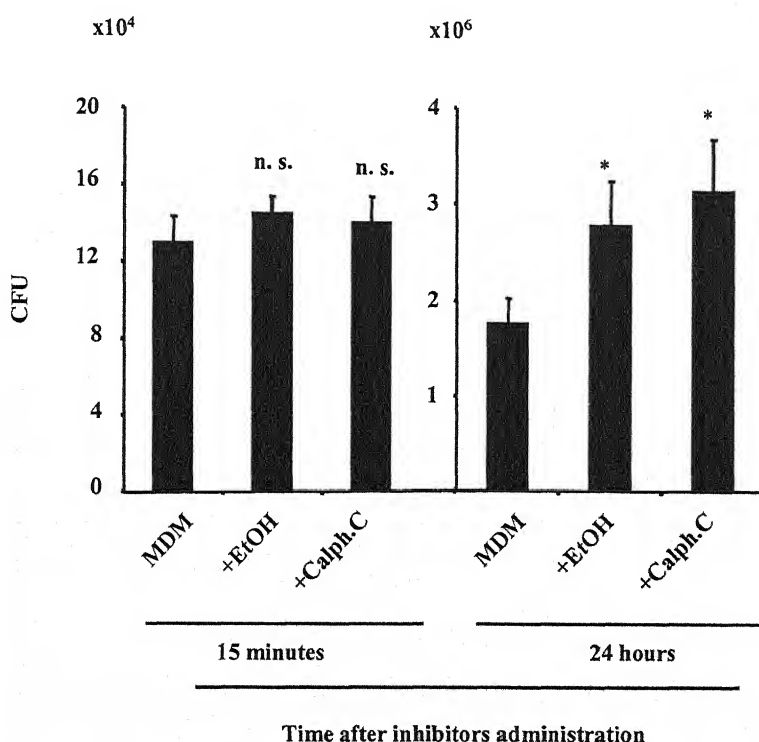
dTHP1 (A) and MDM (B) were prelabelled with [3H]-myristic acid, incubated with 1% ethanol and exposed to either Msm or MTB at the MOI of 1 for 0, 15 and 180 minutes. Pet-OH formation, as an index of PLD activity, was determined by TLC and shown as the percentage of the total radioactivity collected from the individual lane. Each data point represents the means  $\pm$  SEM of values obtained from; Panel A: three independent experiments, conducted in duplicate; Panel B: experiments performed on macrophages derived from three separate donors. Following p values were calculated ;

	dTHP1		MDM	
	15'	180'	15'	180'
$\triangle$ vs. $\diamond$	0.03	0.032	0.02	0.002
$\square$ vs. $\diamond$	0.01	n.s.	n.s.	n.s.
$\circ$ vs. $\diamond$	-	-	n.s.	n.s.
$\triangle$ vs. $\square$	n.s.	0.008	0.01	0.004
$\circ$ vs. $\square$	-	-	n.s.	n.s.

**Inset:** PLD1 expression has been analyzed by western blotting performed in uninfected (lane 1), MTB- (lane 2) Msm- (lane 3) infected MDM (MOI of 1) at 24 hours after infection.

## *Inhibition of PLD activity reduces human macrophages ability to control the growth of Msm*

Macrophages show different capabilities to control the intracellular growth of MTB and Msm (Figure 11), which is associated to different profiles of macrophage PLD activation (Figure 12). To further evaluate the role of macrophage PLD in mycobacterial growth control, Msm intracellular growth obtained after macrophages PLD inhibition with either ethanol [83] or calphostin c [121] at 1 and 24 hours, was compared with mycobacterial replication obtained in time-matched untreated cells. Ethanol and calphostin c were used respectively at the concentration of 0.3% and 10 nM, which were found to be the optimum concentration both for host PLD inhibition as well least effect on MDM viability (data non shown). To avoid any possible interference in the bacterial uptake, macrophages PLD inhibitors were added 3 hours after bacterial exposure. As shown in figure 13, an increase of Msm viability after enzymatic inhibition with either compound was observed.



**Figure 13. Role of macrophage PLD in intracellular Msm growth control.**

MDM were infected with Msm for 3 hours at the MOI of 1. Cells were pulsed or not with either 0.3% ethanol or 10nM calphostin c and further incubated for 1 or 24 hours. Cells were then lysed and intracellular growth of Msm was revealed by CFU assay. Each data point represents the means + SD

of values obtained from the CFU experiments performed on three different donors. Et-OH-MDM compared to untreated cells:  $p=n.s.$  at 1 hour, and  $p=0.01$  at 24 hours; Calph-C-MDM compared to untreated cells:  $p=n.s.$  at 1 hour, and  $p=0.008$  at 24 hours.

In fact, after 24 hours of PLD inhibitor treatment, macrophages exhibited a significant increase of viable Msm when compared to untreated cells.

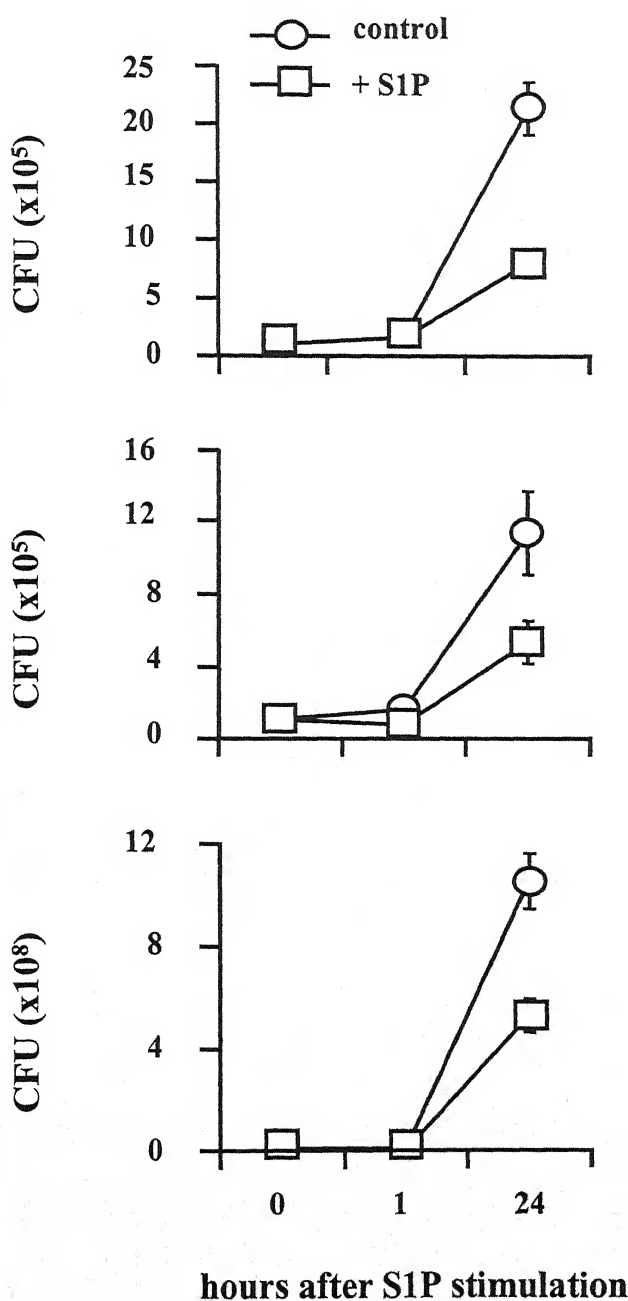
This finding suggests an important role of PLD in the control of non pathogenic Msm intracellular replication.

### ***S1P induces antimycobacterial activity in human macrophages***

As adherent macrophages are permissive for the intracellular growth of non-pathogenic mycobacterial strain Msm [8] and evidences have shown that S1P induces the PLD activity and mobilization of  $\text{Ca}^{++}$  from intracellular pool, which are already known to important phenomenon for anti-microbial activity. We investigated the ability of S1P to directly induce the human macrophages to intracellular growth control of Msm. To this regard, human MDM and dTHP1 cells were infected with Msm for 3 hours as described in methods and mycobacterial viability monitored by CFU assay at 0, 1 and 24 hours after 5  $\mu\text{M}$  S1P stimulation. Results expressed in **figure 14** shows that S1P induces intracellular Msm growth control after infection with both low MOI in MDM (**Figure 14 A**) and dTHP1 cells (**Figure 14 B**) and high MOI in dTHP1 cells (**Figure 14 C**).

In order to see whether S1P is able to induce antimicrobial activity in macrophages against virulent strain MTB H37Rv also, intracellular mycobacterial growth in MDM and dTHP1 cells was analyzed at day 1, 3 and 5 after S1P stimulation. A significant inhibition of intracellular mycobacterial growth was observed after stimulation with 5  $\mu\text{M}$  S1P in both MDM (**Figure 15 A**) and dTHP1 cells (**Figure 15 C**). The analysis performed in terms of dose-dependent response shows a progressive increase of antimicrobial activity which results in a mean of 57% reduction of mycobacterial viability by using the highest dose of S1P and in a mean of 75% of reduction by using either 5 or 50  $\mu\text{M}$  S1P, as determined at day 5 after infection in MDM (**Figure 15 B**) and in dTHP1 cells (**Figure 15 D**), respectively. No direct effect of S1P on mycobacterial viability was observed (data not shown).

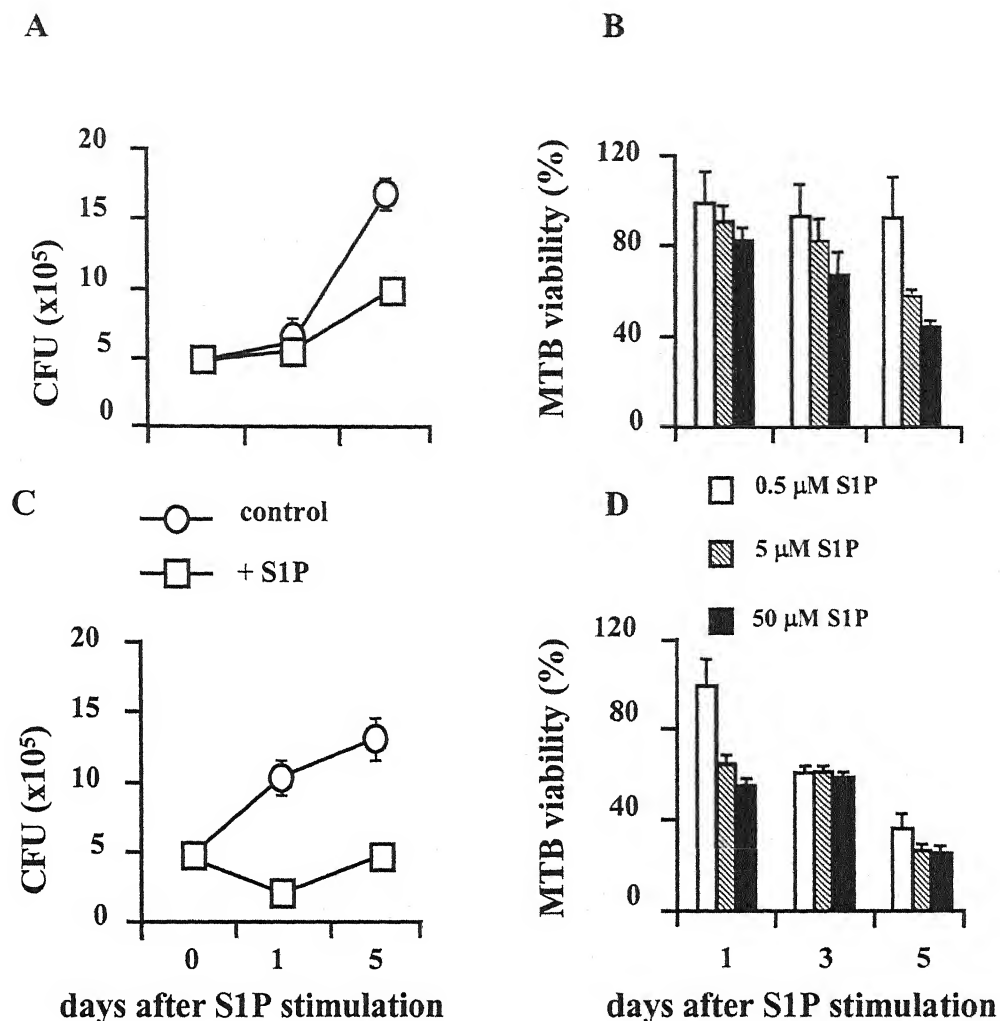
Finally, in order to check the anti-microbial specificity of S1P, THP1 cells were infected with MTB and treated with three different doses (0.5, 5 and 50  $\mu$ M) of Sphingosine, a non-phosphorylated derivative of S1P. At stated time points, CFU assay were performed and colonies were enumerated and compared with MTB infected -non S1P treated and -S1P stimulated cells. **Figure 16** show that S1P but not sphingosine induces antimicrobial activity in macrophages.



**A** **Figure 14. S1P inhibits *M. smegmatis* intracellular growth in human macrophages.**

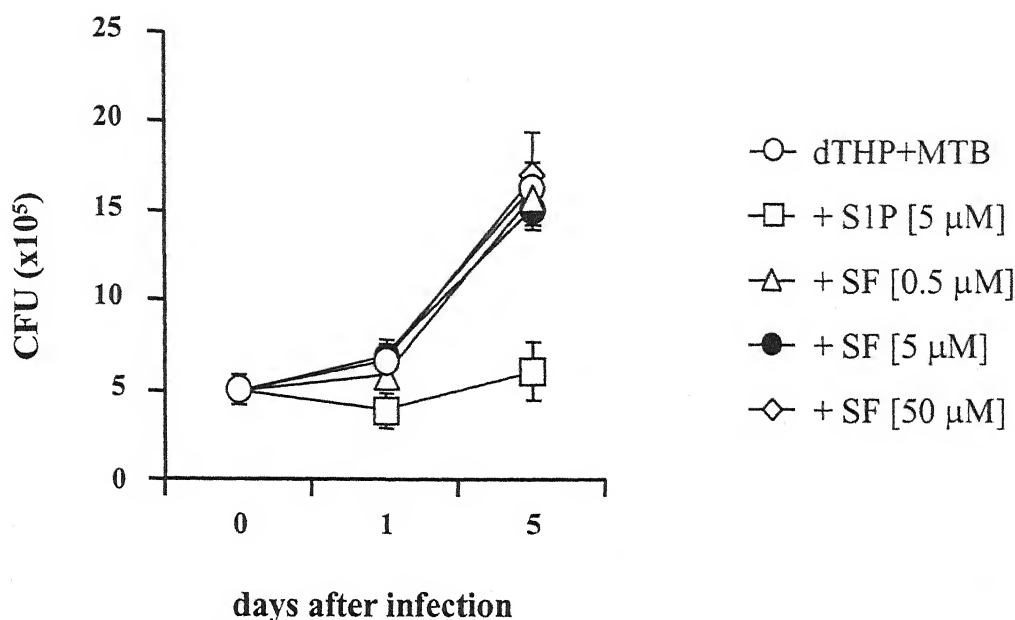
Human MDM were infected at the MOI of 1 (A), dTHP1 cells at the MOI of 1 (B) or 50 (C), and then stimulated with 5  $\mu$ M S1P for 1 and 24 hours. CFU were performed at indicated time points and results are shown as means + S.D. of triplicate values. MDM data are representative of five individual experiments (A), whereas dTHP1 cell data are of three different experiments (B, C). (A)  $p$ =n.s.,  $p$  = 0.002; (B)  $p$ =0.002,  $p$ =0.006; (C)  $p$ =0.001,  $p$ =0.01 vs. Msm-infected control macrophages at 1 and 24 hours after infection, respectively.





**Figure 15. S1P inhibits intracellular MTB growth in human macrophages in a dose-dependent fashion.**

Human MDM (A and B) and dTHP1 cells (C and D) were infected at the MOI of 1 and then stimulated with 5  $\mu$ M S1P for 1 and 5 days (A and C) and with 0.5, 5, 50  $\mu$ M for 1, 3 and 5 days (B and D). CFU experiments were performed and results are shown as means + S.D. of triplicate values. MDM data are representative of five individual experiments (A), whereas dTHP1 cell data are of three different experiments (C). Data are expressed as means + S.D. of the percentages of MTB viability calculated in three separate experiments performed both on different donor-derived MDM (B) and on dTHP1 cells (D). The percentage of MTB viability was calculated as follows: (CFU coming from MTB-infected S1P-treated macrophages/ CFU coming from MTB-infected control macrophages)  $\times$  100. Following p value were calculated; (A)  $p$ =n.s.,  $p$ =0.0002; (C)  $p$ =0.007,  $p$ =0.0002 vs. MTB-infected control macrophages at day 1 and 5 after infection, respectively.



**Figure 16. *S1P* but not sphingosine inhibits intracellular MTB growth in human macrophages.**

dTHP1 cells were infected for 3 hours with MTB at the MOI of 1 and then stimulated or not with 5  $\mu$ M S1P, 0.5, 5, 50  $\mu$ M of Sphingosine (SF) for 1, 3 and 5 days. Cells were then lysed and tested for intracellular growth of MTB by CFU assay. Data were shown as means + S.D. of the triplicate values and are representative of two separate experiments. The following p values were calculated at day 1, 3, and 5 after infection and stimulation in comparison with time-matched MTB-infected dTHP1 cells; 5  $\mu$ M S1P  $p = 0.01, 0.04, 0.0008$  respectively; whereas 0.5, 5, 50  $\mu$ M SF  $p = \text{n.s.}$  at all studied time point.

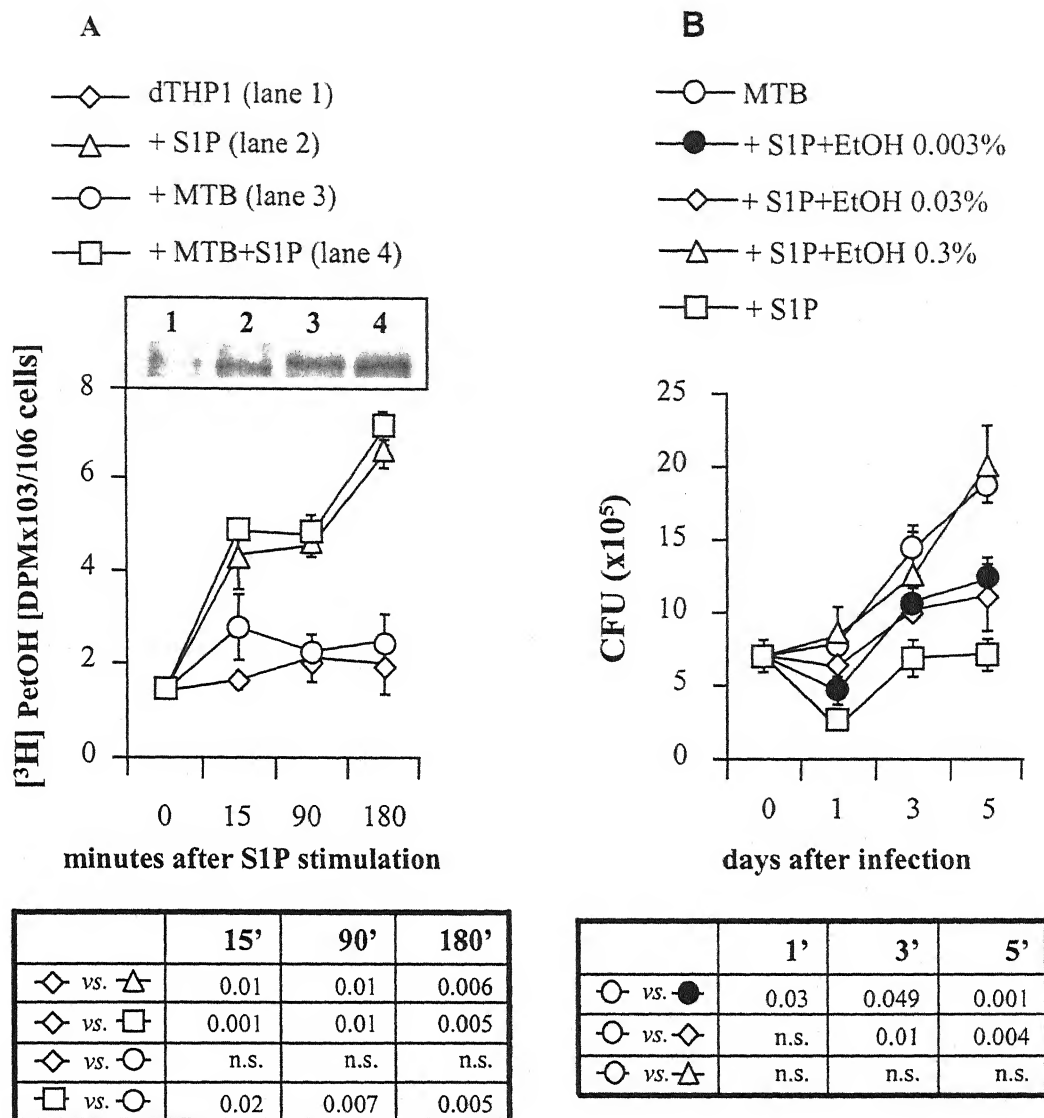
#### ***Macrophage PLD mediates S1P induced antimicrobial activity***

As human macrophage PLD has been previously reported to be involved i) in molecular pathway leading to the activation of antimicrobial mechanisms [78] and, ii) in ATP-induced intracellular mycobacterial killing [41]. Moreover, we have shown -that PLD activity is very crucial for MSm intracellular growth control and -that S1P induces macrophages anti-mycobacterial efficacy against either mycobacteria, involvement of PLD in S1P induced macrophages anti-mycobacterial killing was hypothesized.

To this regard, macrophage PLD activity after MTB infection and S1P stimulation was tested by TLC as described in methods. **Figure 17 A** shows the formation of [ $^3\text{H}$ ]-Pet-OH, as an index of PLD activity, at 15, 90 and 180 minutes post S1P stimulation. In particular, a progressive and significant increase of PLD activity was observed after S1P stimulation in both uninfected and MTB-infected dTHP1 cells when compared to uninfected as well as MTB-infected dTHP1 control, starting from 15 minutes throughout the studied time points.

To see whether higher PLD activity is associated with its higher protein expression, macrophage PLD1 expression was analyzed in dTHP cells at 24 hours after exposure or not with MTB and stimulation or not with S1P. Results reported in **inset of figure 17 A** show higher level of protein expression in all conditions in comparison to dTHP control. In particular, observed results indicate that S1P treatment in both MTB infected or not infected dTHP cells induces the PLD1 expression level which seems to be responsible for higher PLD activity. However in case of MTB infected but not treated cells, higher PLD1 expression was also observed, suggest that expression of PLD1 was not restricted to only S1P stimulation, but could be enhanced after microbial stimuli. Moreover, this up-regulation of PLD1 expression in MTB-infected but not S1P treated cells did not enhance reported basal level PLD activity, which strongly suggest MTB or its component further particulates in the avoidance of PLD activity.

Above results enforce us to further evaluate out idea that PLD inhibition could decrease the macrophage S1P-induced microbicidal capacity. We used a CFU assay to monitor mycobacterial viability in S1P-induced macrophages by inhibiting PLD-mediated PA generation using different concentration of ethanol. **Figure 17 B** shows that ethanol inhibited the S1P-induced anti-mycobacterial activity, in a dose dependent manner, reaching to maximum inhibitory effect at the concentration of 0.3%, resulting in dramatically increase of MTB viability. No direct effect of the used amount of ethanol on MTB growth was observed (data not shown). Similar results were also obtained in course of S1P-induced intracellular Msm growth control (data not shown). All together these results suggest an important role of S1P in macrophage PLD activation resulting in enhanced killing of intracellular as otherwise resistant pathogenic MTB.



**Figure 17. S1P inhibits MTB intracellular growth in human macrophages through a PLD mediated mechanism.**

(A) dTHP1 cells were labeled with 3H-myristic acid, infected with MTB at the MOI of 1 and stimulated with 5  $\mu$ M S1P for 15, 90, 180 minutes. Phosphatidylethanol (PetOH) formation, as an index of PLD activity, was determined by TLC and shown as DPM values. Data are expressed as means + S.D. of the triplicate values from one representative experiment of three. (Inset A) PLD expression has been then analyzed by western blotting in uninfected (lane 1), S1P-treated (lane 2), MTB-infected (lane 3), MTB-infected S1P-treated (lane 4) dTHP1 cells at 24 hours post incubation.

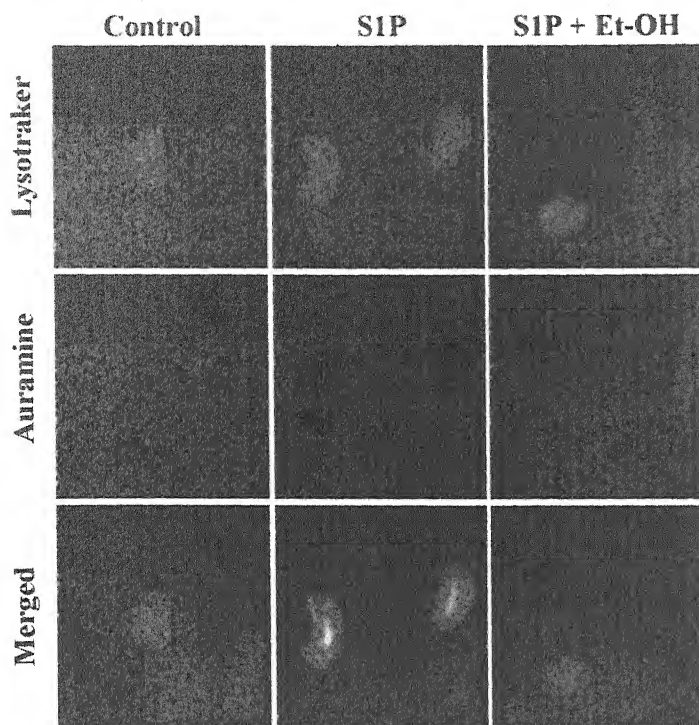
(B) dTHP1 cells were infected with MTB at the MOI of 1 and incubated for 1, 3 and 5 days with 5  $\mu$ M S1P together with the indicated amounts of ethanol (Et-OH). CFU were shown as means + S.D. of the triplicate values and are representative of three separate experiments.

### ***S1P favors acidification of MTB containing phagosomes***

As macrophage PLD is involved in phagolysosome biogenesis [41], and MTB is known to reside in nonacidified vacuoles sequestered away from late endosomal compartments, the acidification of MTB containing phagosomes in S1P-stimulated cells was investigated by confocal fluorescent microscopy (**Figure 18 A**). As evident in **figure 18 A**, in absence of S1P, phagocytosed MTB bacilli appeared green, indicating their presence in non-acidic vesicles separate from the red stained acidic vacuoles. In contrast, S1P treatment induces a significant increase in the acidification of MTB-containing phagosomes, which appeared yellow, indicating the colocalization of green bacilli in acidified phagosomes. To determine the involvement of PLD signaling in S1P induced phagosome-lysosome maturation, MTB infected-S1P stimulated macrophages were simultaneously treated with ethanol. The addition of ethanol together with S1P almost completely reverted the S1P induced MTB containing phagosome maturation. Finally, the percentage of MTB residing in acidic phagosomes over total intracellular mycobacteria was expressed after serial observations (**Figure 18 B**).

### ***S1P induces up-regulation of late endocytic markers***

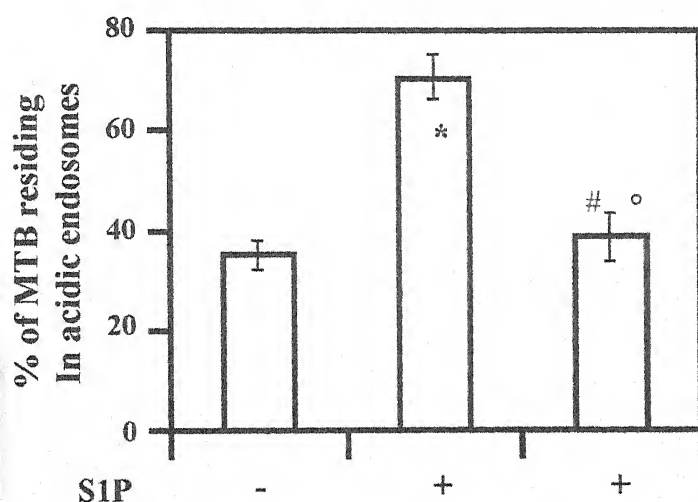
As MTB is known to reside in nonacidified vacuoles sequestered away from late endosomal compartments, as a part of their intracellular survival strategy, the evidence by above confocal microscopy experiments, that S1P favors acidification of MTB containing vacuoles suggests that phagolysosome maturation process was in progress. In this context, to make more convincing our confocal microscopy results, we pursued the western blotting analysis to distinguish any modulation of late endocytic enzymatic markers.



**Figure 18.** *MTB co-localizes in lysosomes after S1P stimulation.*

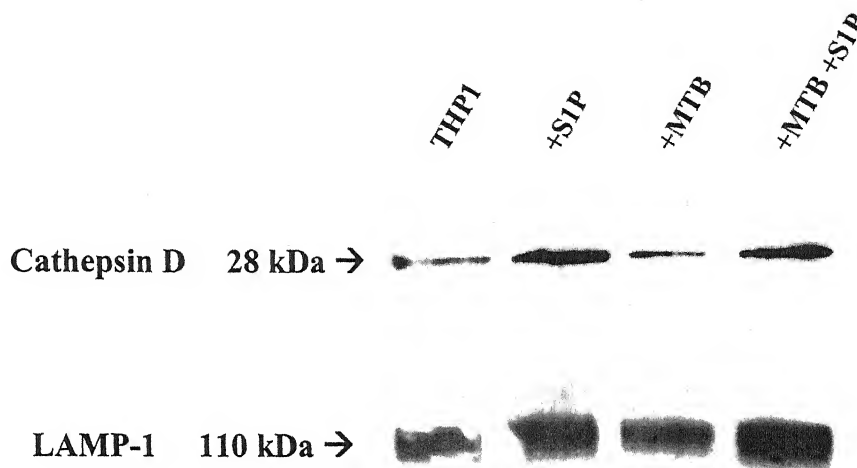
(A) dTHP1 cells were labeled with LysoTracker Red (red, to detect lysosomes), infected with MTB at the MOI of 1, incubated for 3 hours with 5  $\mu$ M S1P in the presence or not of 0.3% ethanol (Et-OH). Cells were then fixed, permeabilized, and beaded on confocal slides followed by staining with auramine (green, to detect mycobacteria). Finally, cover slips were mounted with

vectashield mounting medium and edges were sealed with nail polish. Yellow appearing MTB was said to be colocalized with lysosomes. (A) Representative picture from three separate experiment shows the increase of MTB colocalizing with lysosomes after S1P treatment and the reverse effect exerted by ethanol.



(B) Summary of the mean percentage + SEM of MTB-colocalized with lysosomes, determined by counting over 150 bacilli from at least 60 macrophages per sample. Three different experiments were assessed. \*  $p < 0.0001$  and °  $p = n.s.$  vs. MTB-infected control cells.

To this regards, dTHP1 cells were infected or not for 3 hours with MTB and further stimulated or not with 5  $\mu$ M S1P for 24 hours. Cells were then lysed by suspending in lysis buffer and total proteins were separated onto SDS-PAGE, followed by immunoblotting with anti LAMP-1 and anti-Cathepsin D antibodies (BD transduction laboratories). Results reported herein suggest that S1P treatment increased the expression of Cathepsin D significantly, while MTB had the same level of expression, in comparison to non infected dTHP1 control (Figure 19).



**Figure 19. *S1P induces up-regulation of Cathepsin D and LAMP -1.***

dTHP1 cells were infected or not for 3 hours with MTB and further stimulated or not with 5  $\mu$ M S1P for 24 hours. Cells were lysed by suspending in SDS lysis buffer. 15  $\mu$ g total protein was separated onto SDS-PAGE (8% for LAMP-1, 12% for Cathepsin D (w/v) gel) and immunoblotted with anti-LAMP-1 and anti-Cathepsin D antibodies. Signal was then developed by using ECL substrate for HRP and exposed to CL-X-posure film (Pierce, UK).

LAMP-1 expression was also up-regulated by S1P stimulation, which was highest where the phagosomes were generated by ingestion of MTB (Figure 19). MTB infected but not stimulated cells also showed a bit higher but not significant higher level of LAMP-1 expression, suggest that some population of MTB containing phagosome may also migrate to maturation pathway.

### ***S1P reduces mycobacterial outgrowth and granulomatous response in infected mice***

In order to test the antimicrobial efficacy of S1P *in vivo*, we performed experimental infection in mice either with Msm or MTB. Ten fold reduction of mycobacterial burden was observed in the lung of Msm-infected mice (**Inset of Figure 20**) at the intermediate dose of 5 nmoles / mouse.

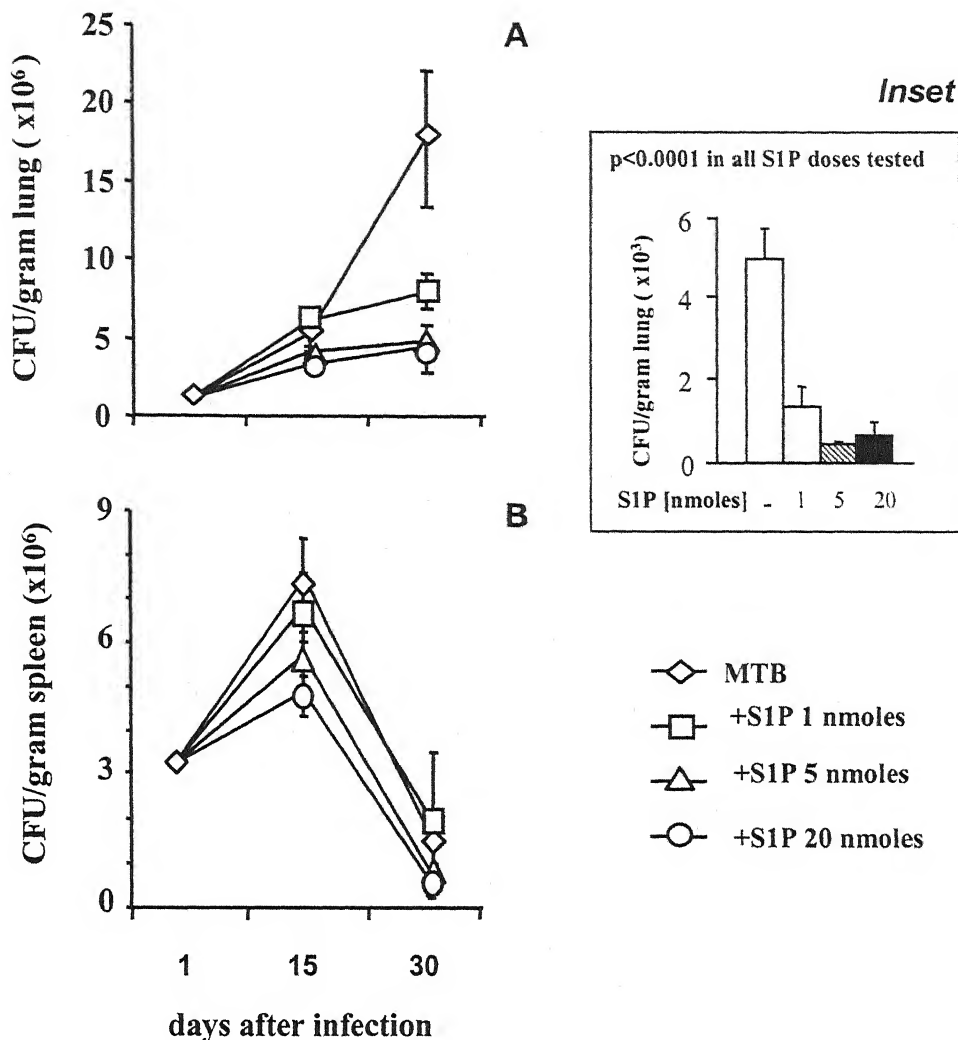
Moreover, S1P treatment reduced MTB burden in a dose dependent manner both in the lung (**Figure 20 A**) and in the spleen (**Figure 20 B**), reaching about 47% mycobacterial growth inhibition after two consecutive treatment of 20 nmoles S1P/mouse in 7 days of interval. However, when treatment was prolonged for other 2 consecutive doses with same concentrations, bacterial outgrowth were inhibited for 75% in both organs (**Figure 20 A**) (**Figure 20 B**).

Histological analysis shows an extensive granulomatous reaction with caeseous necrosis in the lungs of MTB-infected mice (**Figure 21 A**) characterized by a mixed neutrophil, macrophage, and lymphocyte infiltrate (**Figure 21 E**) and extensive mycobacterial burden (**Figure 22 A, C**).

A significant lower granulomatous reaction, with no evidences of necrosis (**Figure 21 B**), characterized by a prevalent macrophage infiltrate (**Figure 21 F**) and a highly significant reduction of auramine-stained mycobacteria (**Figure 22 B, C**) was observed after treatment with S1P at the highest dose of 20 nmoles/mouse.

The quantitative analysis of tissue damage showed a dose dependent recovery of pulmonary parenchyma which increased up to 3 fold after treatment with the highest dose 20 nmoles/mouse, corresponding to about 50% recovery of the total pulmonary area analyzed (**Figure 21 G**).





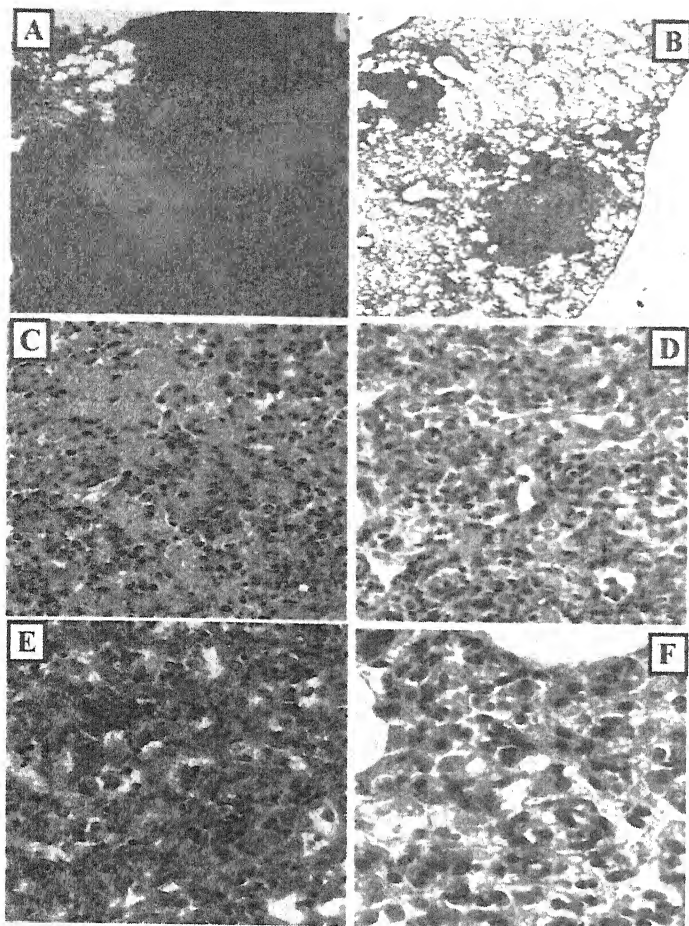
**Figure 20. S1P reduces mycobacterial load in the course of in vivo mycobacterial infections.**

Mice were infected with 106 Colony forming units (CFU) of either *M. smegmatis* or MTB H37Rv and treated or not with 1, 5, 20 nmoles S1P. The scheme of treatment was followed as described in methods.

At day 3 post S1P treatment lungs (*inset*) from *M. smegmatis*-infected mice; at day 15 and 30 post S1P treatment lungs (A) and Spleen (B) from MTB-infected mice were sterily collected, homogenized and CFU were performed in triplicates. Data are expressed as means + SD of

	Lungs		Spleen	
	15	30	15	30
◇ vs. □	n.s.	0.001	0.01	n.s.
◇ vs. △	0.01	>0.001	0.001	0.01
◇ vs. ○	0.001	>0.001	>0.001	0.01

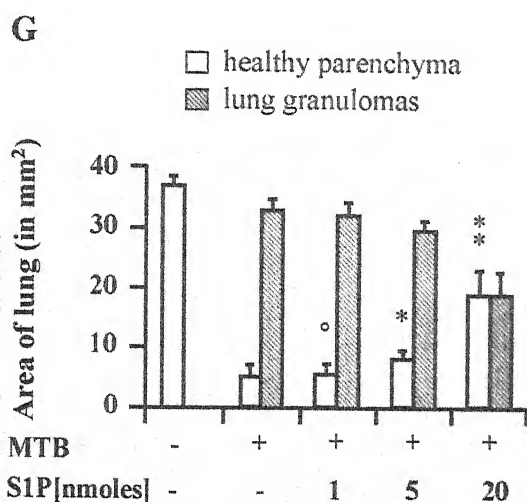
the CFU collected from 4 different mice per group.  $p < 0.0001$  for S1P doses tested for Msm-infected mice (*inset*) whereas following  $p$  values were calculated vs. S1P-untreated MTB-infected mice.



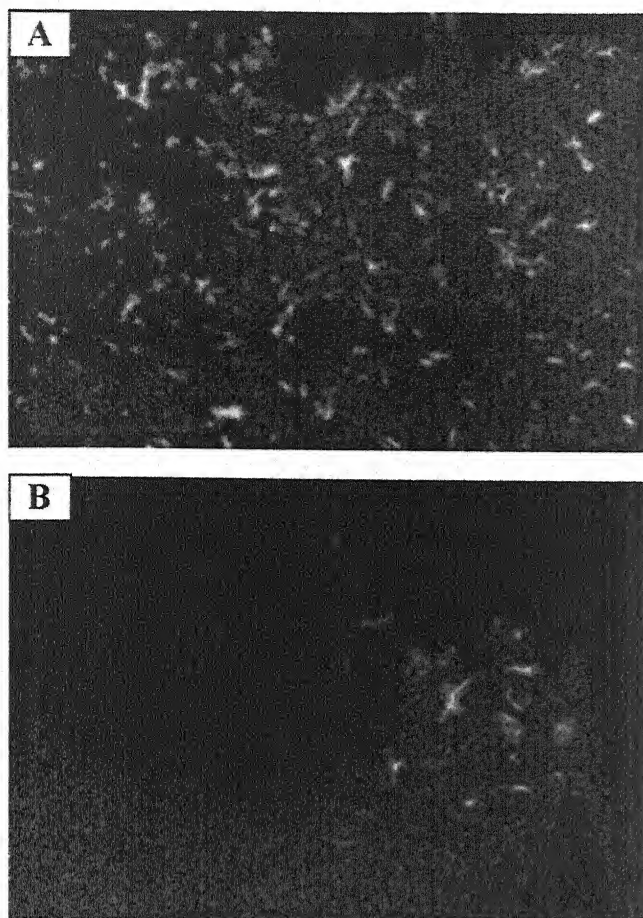
**Figure 21.** *Morphological examination of lung tissue in MTB-infected mice (A, C, E) and in MTB-infected SIP-treated mice (B, D, F).*

Lung specimens were fixed, paraffin embedded and stained with hematoxylin and eosin and examined for granuloma formation. In particular, almost all the lung parenchyma was occupied by granulomas in MTB-infected mice (A, magnification 200x) which were characterized by a necrotic core, a polymorphous inflammatory infiltrate (E, magnification 800x). SIP treatment induced a dose dependent reduction of lung

granulomas (B, magnification 200x) which were characterized by a prevalent macrophage infiltrate without a significant necrotic reaction (F, magnification 800x).

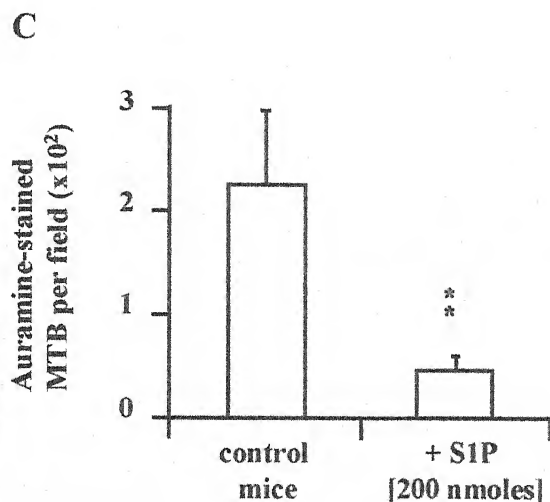


**(G)** Summary of the areas of healthy lung parenchyma (empty column) and of granulomas (dashed column), expressed as means + S.D., evaluated by analyzing 10 fields covering the entire lobe surface coming from 4 mice per experimental group.



**Figure 22.** *In situ* staining of MTB present in lung tissue of MTB-infected mice (A) and in MTB-infected S1P-treated mice (B).

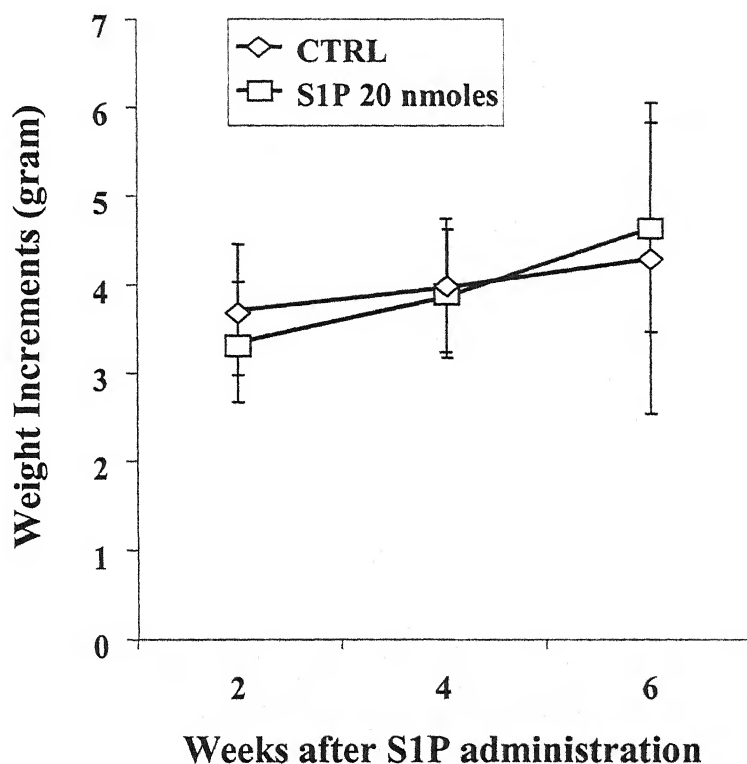
Slides of lung tissues were prepared and stained with auramine stain of "TB fluorescent stain kit M" (Becton Dickinson, MD) as per the instructions of manufacturers. Representative picture hereby shows disseminated auramine-stained mycobacteria (A, magnification 2000x) in MTB infected lung granuloma, whereas, treatment reduces the number of mycobacteria hence auramine-stained less number of mycobacteria (B, magnification 2000x).



**(C)** Summary of the means + S.D. of the number of auramine-stained bacilli observed on 20 granulomas per experimental group by using Scion Image Package system software. ° p=n.s., \* p=0.02, \* \*p<0.0001 vs. MTB-infected S1P-untreated mice.

### *S1P doesn't show any sign of toxicity in utilized animal murine model*

As a preliminary toxicology's studies, *in vivo* toxic affect of S1P was done by monitoring and comparing the body weight of mice administered with 20 nmoles of S1P with those of mice received physiological solution. After first 20 nmoles of intravenous S1P inoculation, following once a week for a total of 6 weeks, body weight of S1P received mice weren't different in comparison to age-matched physiological solution received control mice (Figure 23). Another data which confirm the non toxicity of S1P is that animals received the molecules were lived normally for their full life span, when compare with non treated mice group. (data not shown).

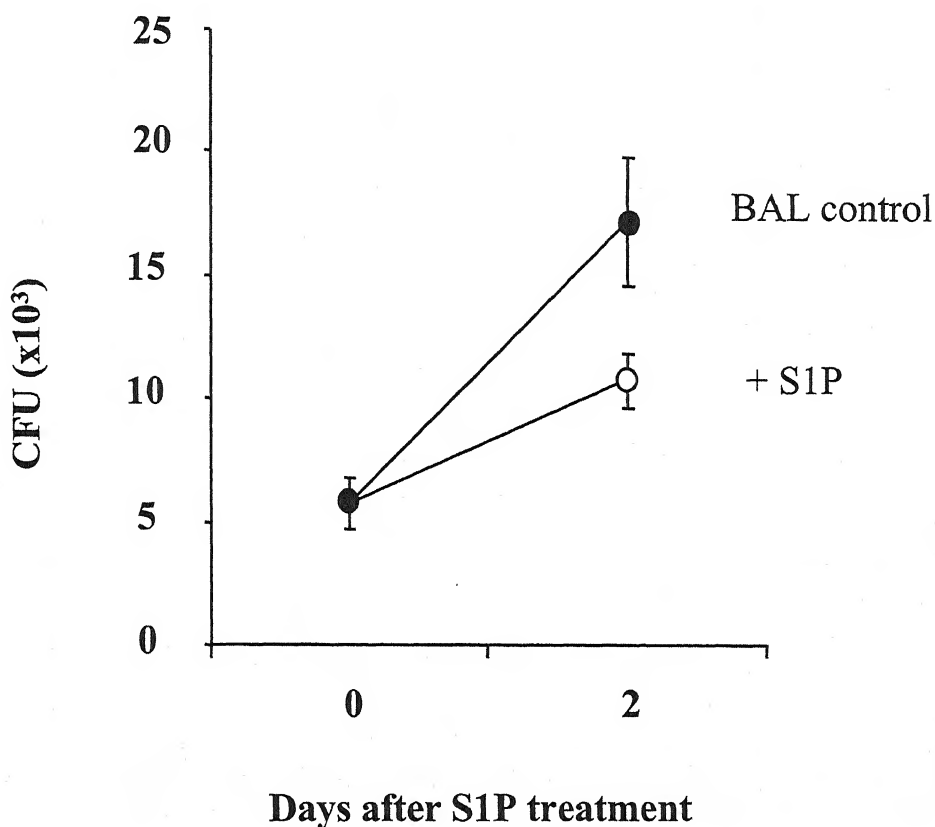


**Figure 23. Body weight monitoring of BALB/c mice.**

Mice utilized for *in vivo* experiments were checked for body weight gain/loss at 2, 4 and 6 weeks of S1P administration or not and compared with physiological solution received mice. Data are expressed as means + SD of the body weight recorded from 8 different mice per group.

***S1P induces the ex vivo anti-mycobacterial activity in BAL from patients with tuberculosis***

In order to verify *ex vivo* anti-mycobacterial efficacy of S1P, alveolar macrophages from patients with tuberculosis were isolated and cultured with or without 5  $\mu$ M S1P and CFU analysis was done as described in methods. Result hereby demonstrates that S1P stimulation significantly reduces about 40% mycobacterial load in alveolar macrophages of pulmonary TB patients in comparison to non-treated control (Figure 24).



**Figure 24. *S1P induces the ex vivo anti-mycobacterial activity in BAL from patients with tuberculosis.***

Alveolar macrophages isolated from BAL of tuberculosis patients were stimulated or not with 5  $\mu$ M S1P and cultured for 0 and 2 days. Data are expressed as means + S.D. of the all CFU values collected from five different patients each performed in triplicates.  $p > 0.0001$

## ***Macrophage proteome after S1P treatment reveal up regulation of 20 different proteins***

In order to identify the S1P induced macrophage molecule/s involved in mycobacterial killing total macrophage proteome was prepared by utilizing two dimensional gel electrophoresis followed by preliminary mass spectrometric and database analysis. In this context cells were infected or not with MTB at MOI of 1 for 3 hours and then further incubated for 24 hours after stimulation or not with 5  $\mu$ M S1P. At the end of the incubation cells were collected, washed, lysed, proteins were purified and resolved on 2D-PAGE followed by staining and image acquisition as described somewhere in the methods. All the proteins extracts were prepared from duplicate cultures and subjected to two-dimensional polyacrylamide gel electrophoresis. **Figure 25** displays the macrophage proteomes obtained after various conditions (such as infected or not and stimulated or not) at same time kinetics utilized. In each sets of experiments dTHP cells infected with MTB and treated with S1P (dTHP1+MTB+S1P) was considered as reference gel (master gel) and was utilized for comparative analysis throughout the set (**Figure 26**). Image analysis revealed the presence of ~1700 different proteins within the pH 3-10NL and MW range of ~12 to ~100 kDa. A total of 21 different proteins (SSP number; 1106, 2003, 2005, 2630, 3116, 3115, 4101, 4109, 4118, 4122, 4428, 5105, 5402, 6102, 7018, 7522, 8014, 8017, 8020, 8032, 8114, 8138, ) were found to be upregulated after S1P treatments and among these some proteins (SSP number; 8020, 8032, 8114, 8138) were found to be exclusively expressed in the presence of infection and treatment (dTHP1+MTB+S1P). Reference spot numbers, together with identities or SWISS-PROT entry names (from: <http://us.expasy.org>), whenever available, are superimposed upon the image. Spots that changed upon S1P treatments are indicated in the proteome map with their spot numbers ('i.e.' SSP number). PDQuest<sup>R</sup> image analysis software version 6.0 (Bio-Rad) was used to detect and quantify the volume of all the spots. Histograms obtained from each spot were examined for S1P treatment related changes in the protein expression levels. In general at least a two-to three fold difference in protein expression levels between S1P treated and untreated could be reliably detected and considered as significant.



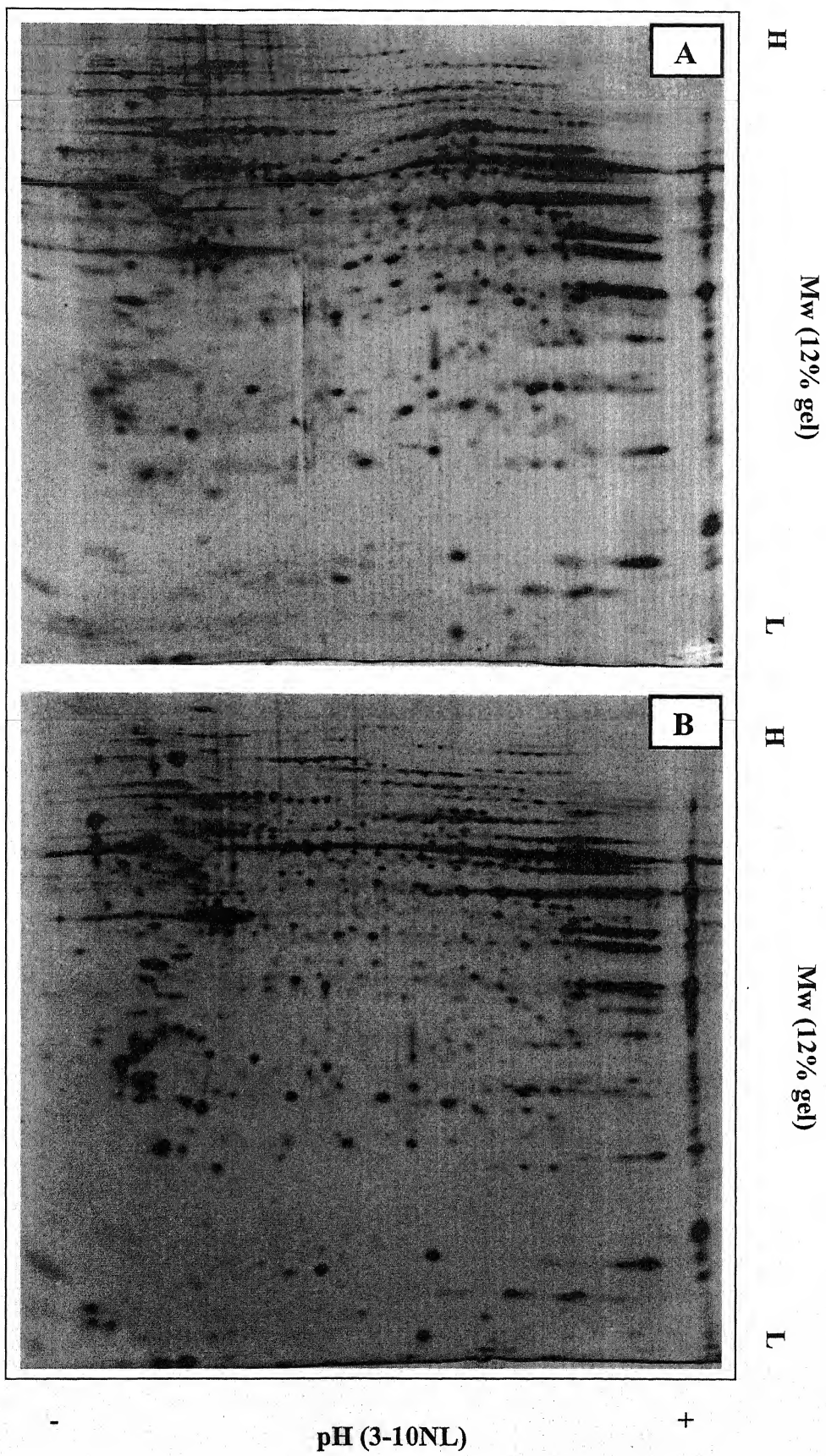


Figure 25. *dTHP1* proteome

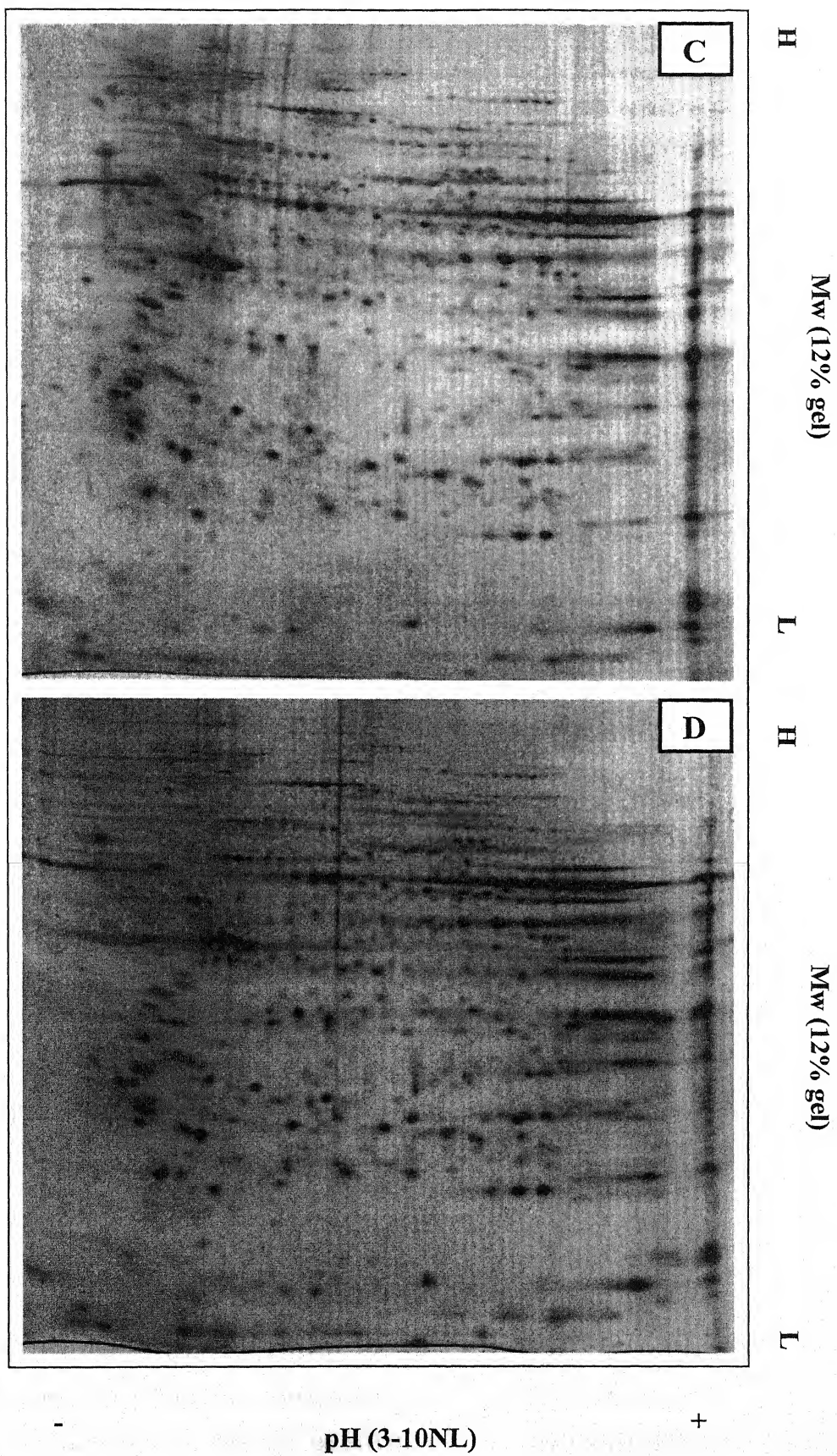
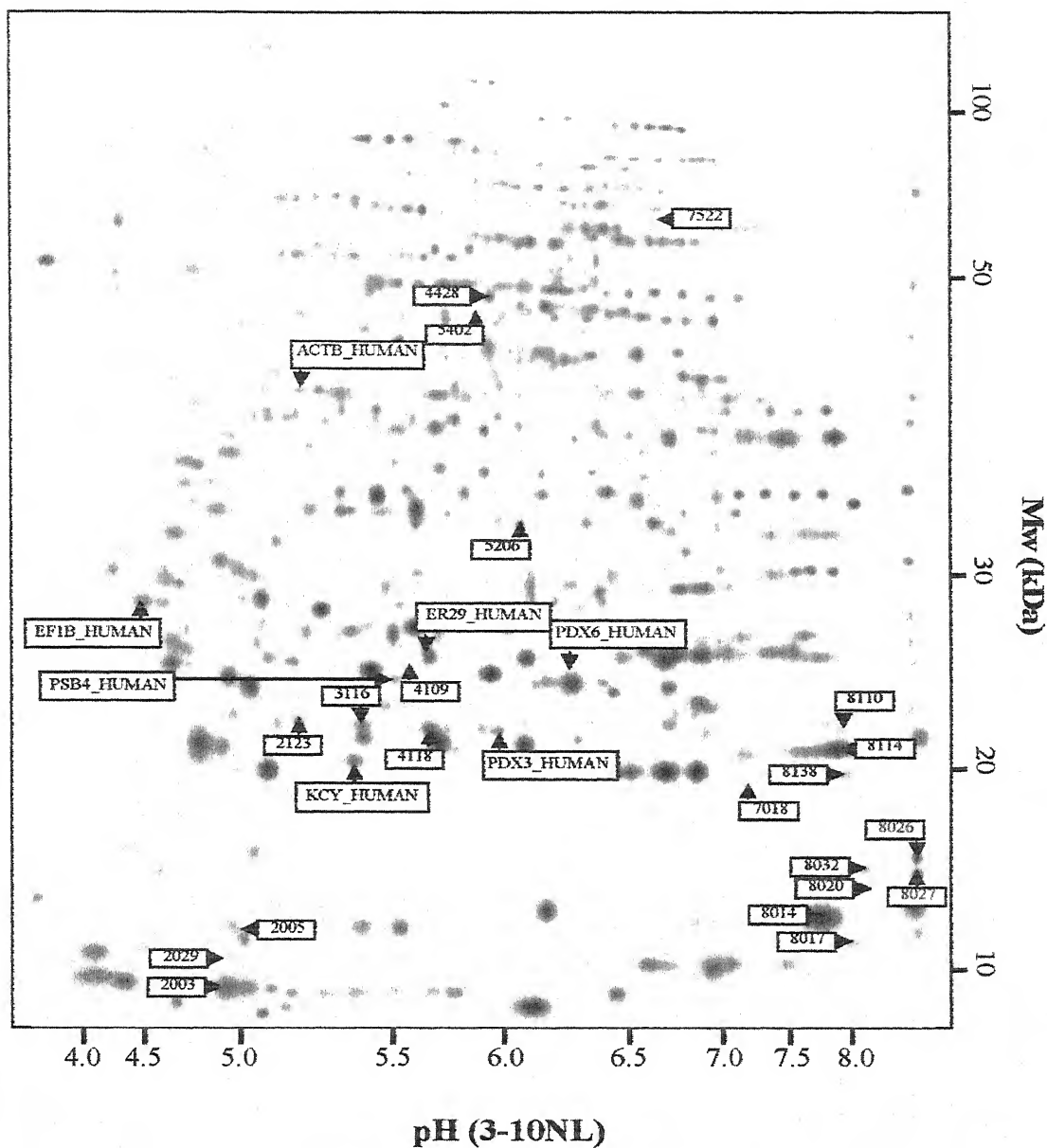


Figure 25. *dTHP1* proteome



**Figure 25. dTHP1 proteome.**

Cells were infected or not with MTB and stimulated or not with SIP for 24 hours. Proteins were extracted from each cells condition and 2D was performed. Slave gel from each condition (A) dTHP1, (B) dTHP1+SIP, (C) dTHP1+MTB, (D) dTHP1+MTB+SIP is shown as a representative figure.



**Figure 26. Reference gel (master gel)**

dTHP1+MTB+SIP (Figure 25 D) Slabe gel was used as reference gel and was utilized for comparative analysis to see the SIP stimulated protein changes. Spots that changed upon SIP treatment are indicated with their spot number, and from these, the ones identified by means of mass spectrometry are annotated with SWISSPROT entry names (XXXX\_HUMAN).

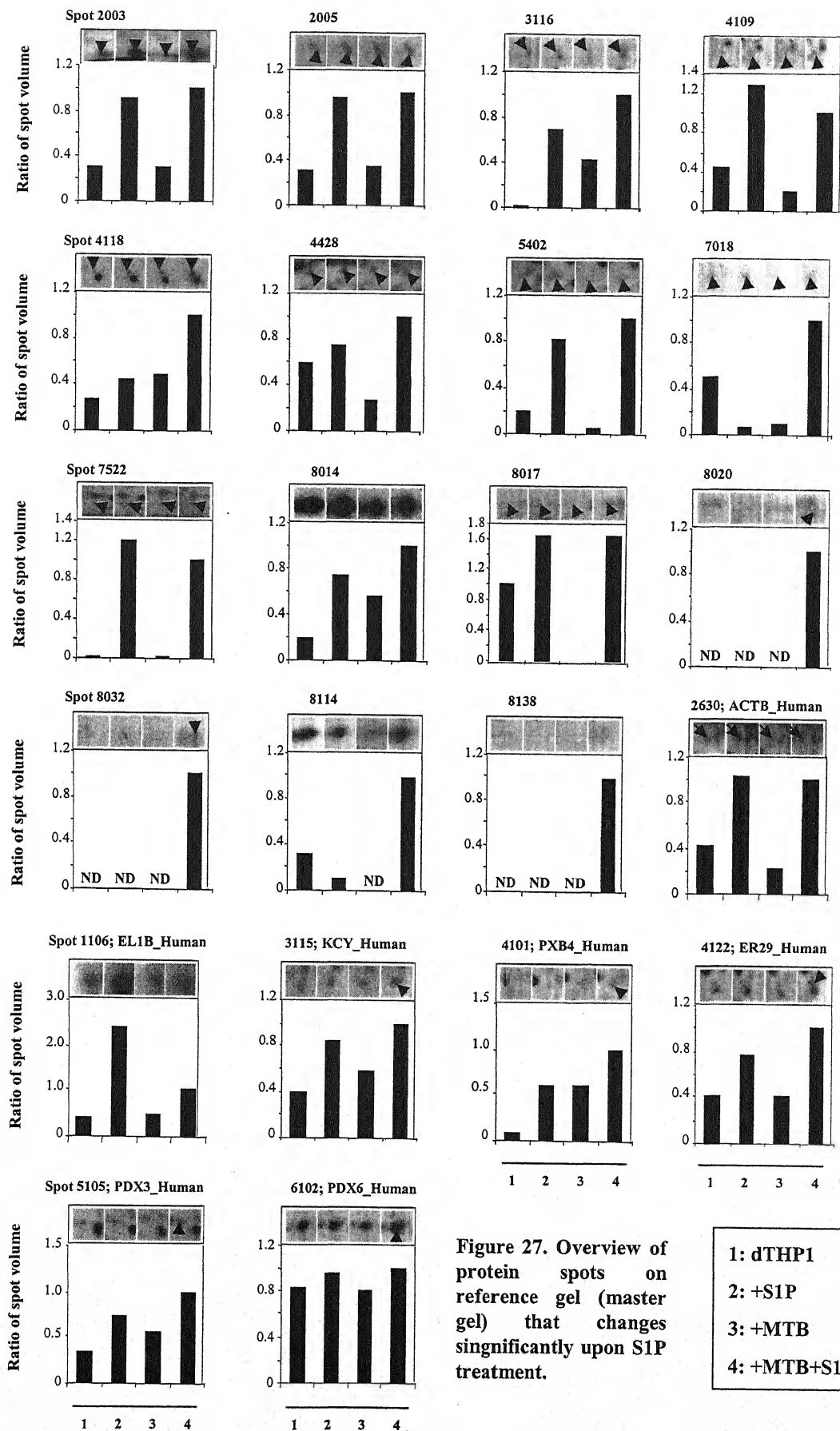
Difference in the spot volume for those 21 spots from which statistically significant differences were observed among various experimental conditions used is shown as histograms (**Figure 27**). In order to identify these 21 proteins, peptides were extracted after trypsin-digestion of the gelplug and were subjected to MALDI-TOF-MS.

Protein identities are indicated whenever the identification was successful. Representative spot images are displayed on the top of each histogram (**Figure 27**). Twelve out of twentyone protein spots which changed upon S1P stimulation were not identified, because of the fact that peptides derived from multiple proteins were present in one spot or because of low peptide fragment ionization, compared to background signal, or because of presence of higher keratine contamination signal comparison to peptides of interest signals. However, these unidentified peptides and results are being confirmed further on MALDI-TOF-MS after troubleshooting of possible existing problem.

Spot number 2630 was identified as human  $\beta$  actin protein (SWISS PROT accession number: P60709; ACTB\_HUMAN), which was upregulated significantly in presence of S1P stimulation (**Figure 29 A**).

Spot number 4118 was also upregulated after S1P treatment and was identified as Integrin,  $\beta$ -like 1 with EGF-like repeat domains (SWISSPROT accession number: Q96QX7; Integrin, beta-like 1 (With EGF-like repeat domains; Fragment)) (**Figure 29 B**).

Spot number 8114 was identified as MHC class II antigen with 85.2% sequence coverage, (SWISSPROT accession number: Q8SNCO), which was not detected (ND) in case of infection only, whereas was over expressed significantly after S1P treatment. Moreover, in absence of bacilli S1P treated or not treated dTHP cells show basal level of expression (**Figure 29 C**).



**Figure 27. Overview of protein spots on reference gel (master gel) that changes significantly upon SIP treatment. (For graph please see the next page)**

Each graph represents the average spot volume determined from duplicate cultures. Representative gel images are displayed on the top of each graph. Identified proteins are indicated. ND: Spot not detected by the software.

**Table 9. Summary of expression data and identified proteins.**

Spot Number	SWISS-PROT Accession number	SWISS-PROT Entry name	Method of identification	% sequence coverage	Theoretical Mw/PI	Experimental ~Mw/PI
2630	P60709	ACTB_HUMAN	MALDI	18.4%	41605.54/5.29	42000/5.3
1106	P24534	EF1B_HUMAN	MALDI	17.1%	29603/4.54	29000/4.5
3115	P30085	KCY_HUMAN	MALDI	21.2%	21510/5.41	20000/5.45
4101	P28070	PSB4_HUMAN	MALDI	11.3%	24306/5.50	24000/5.52
4122	P30040	ER29_HUMAN	MALDI	24 % (of precursor seq.)	26862/5.55	26000/5.57
5105	P30048	PDX3_HUMAN	MALDI	23.7%	23641/6.02	23000/6.0
6102	P30041	PDX6_HUMAN	MALDI	31.1%	25812/6.28	25000/6.2
8114	Q8SNC0	MHC class II antigen (fragment)	MALDI	85.1%	ND	22000/7.55
4118	Q96QX7	Q96QX7	MALDI	41.3%	ND	22000/5.7

Theoretical pI and Mw were calculated using the SWISS-PROT compute pI/Mw tool ([http://www.expasy.au/tools/pi\\_tool.html](http://www.expasy.au/tools/pi_tool.html)) on available amino acid sequences derived from SWISS\_PROT annotated human proteins

## Discussion

The dynamic interaction between MTB and human macrophages plays a key role in determining the outcome of infection. In this context, the identification of physiological ligands and the elucidation of the molecular mechanisms leading to the mycobacterial intracellular growth control represent an important goal for tuberculosis research. In fact, no physiologic agonists have yet been reported that can unambiguously stimulate the anti-tuberculosis activity of macrophages both *in vitro* and *in vivo*, although conflicting data regarding several cytokines, immunomodulators and other inflammatory mediators have been presented. Moreover, the specific signaling pathways that regulate, and the effector mechanisms that mediate, the killing of intracellular MTB are poorly elucidated [46]. The present study reports a novel role of S1P as a regulator and PLD as a mediator of antimicrobial activity, exerted both *in vitro* and *in vivo* against non-pathogenic Msm as well as pathogenic MTB infection. Interestingly, the results reported herein show that dTHP1 are more sensitive to S1P signaling in terms of both dose-dependent responsiveness and mycobacterial viability reduction in comparison with MDM. This evidence may be related to different expression of S1P receptors in either host cells and such possibility is currently under investigation. The experimental use of Msm, as an “absurd” model for tuberculosis research, has been recently debated [108]. It may survive and multiply inside adherent macrophages [8]. As Msm is fast-growing and transformable, is being used as a host to manipulate and express MTB and *M. avium* genes [50] still retaining unique mycobacterial determinants. The comparative *in vitro* and *in vivo* analysis of S1P-induced antimicrobial effect against both mycobacteria supports the idea that fast growing mycobacteria, such as Msm, may represent a useful experimental model to get preliminary and rapid information regarding the molecular mechanisms involved in macrophage antimycobacterial activity.

The present results demonstrates that in the course of *in vitro* infection, Msm induces higher levels of macrophage PLD activity than MTB, and this enzymatic activity is involved in its intracellular growth control. As a number of MTB pathogenic mechanisms have already been reported that include avoidance of generation of ROI and RNI, down-regulation of HLA-class II,

and inhibition of phagolysosome maturation [46], our study may provide another possible escape mechanism exerted by MTB while inside intracellular compartments. In this context, as PLD has been reported to be involved in ROI generation [7] and in phagolysosome fusion [77], the avoidance of its activation can represent an upstream escape mechanism from the hostile macrophage environment, and hence contributing to the intracellular MTB survival. Moreover, in macrophages, heat-killed MTB does not induce significantly different PLD activation than those in live MTB, suggesting the absence of an active mechanism exerted by MTB to avoid PLD activation, but some pre-constituted element which should be differently expressed in MTB and Msm. These data are coherent with previously reported results by Locati *et al*, showing that non-pathogen-derived AraLAM induces higher levels of PLD1 activity than pathogen-derived ManLAM [84]. However, the results reported herein show that the observed differences in PLD activity are not related to differences in PLD protein expression in course of infection with either mycobacteria, suggesting that PLD activity can be, in turn, metabolically controlled. The direct contribution of PLD in intracellular Msm killing was also confirmed by utilizing ethanol [83] or calphostin-C [121] as inhibitors of PLD. In this regard, ethanol, a short chain primary alcohol, inhibits PLD-mediated generation of PA by substituting for water as the nucleophilic acceptor of the phosphatidyl moiety, deviating the reaction towards the formation of the metabolically inactive Pet-OH. Thus, ethanol inhibits PLD-dependent responses without blocking enzyme turnover, whereas calphostin-c affects the productivity of reaction by binding irreversibly at the catalytic domain of the enzyme.

As we have shown that PLD activity is associated with a different ability of macrophages to control pathogenic or non-pathogenic mycobacteria and exogenous S1P has already been reported to induce PLD activity leading to the formation of intracellular PA [35] which is involved in the generation of a number of macrophage antimicrobial activities, such as phagocytosis [78], ROI production [55], intracellular trafficking of endocytosed immunocomplexes to lysosomes [93], and phagolysosome maturation in the course of ATP-induced mycobacterial killing [41]. On these grounds, the role of PLD in S1P-induced

antimicrobial activity was hypothesized. The results reported herein show that S1P-induced antimicrobial activity involves activation of macrophage PLD which favors progressive acidification of mycobacteria containing phagosomes and MTB killing. Support for a key role of PLD in S1P-induced mycobacteriocidal activity in human macrophages consisted of elevation of [ $^3\text{H}$ ]-Pet-OH formation, as metabolically stable index of PLD activity, and concordant inhibition of mycobacterial killing by using ethanol, as specific inhibitor of PLD-mediated PA generation. Finally, the role of PLD-mediated phagolysosome maturation in the course of S1P-induced mycobacterial growth control was analyzed. In this context, the molecular probe LysoTracker Red is a weak base that is used to study phagosome acidification because it is selectively taken up within cells by acidic organelles. In the present study, LysoTracker Red targeted MTB-containing phagosomes following S1P stimulation, suggesting that phagolysosome maturation was progressing. It is still not known whether PA, a known fusogen to endosome membrane [69], can directly promote endosome membrane fusion or if the effect is mediated by some other downstream effector mechanisms. In fact, PLD may also activate additional phospholipases, such as  $\text{PLA}_2$ , which can favor endosome fusion through the generation of arachidonic acid [89]. To this regard, evidences have been reported showing that S1P may induce arachidonic acid formation through a signaling pathway involving EDG receptors, PLD and  $\text{PLA}_2$  [142].

The success of MTB as a pathogen resides in its ability to persist and replicate within the hostile environment of macrophages. A number of pathogenic mechanisms have been reported to contribute to intracellular mycobacterial survival which include avoidance of ROI and RNI generation, HLA-class II down-regulation [46], and blockage of both  $\text{Ca}^{++}$  signaling and phagolysosome maturation via sphingosine kinase inhibition [87]. Furthermore, it has been reported that stimulation of EDG receptors by exogenous S1P induces intracellular S1P formation leading to release of  $\text{Ca}^{++}$  from internal stores [95]. In this context, the results reported herein show that the S1P-induced elevation in PLD activity is not related to higher differences in PLD1 protein expression, suggesting that PLD activity can be in turn metabolically controlled, as requirement of  $\text{Ca}^{++}$  for PLD activation has been reported [126]. Thus, it is possible to



hypothesize that the addition of S1P may restore required signaling, leading to PLD activation and phagolysosome maturation. Although further investigations are needed to deeply assess the molecular pathways involved in S1P-induced mycobacterial killing, the results reported herein support the idea that PLD can play a key role in macrophage-mediated antimycobacterial activity.

S1P has been shown to play a protective role in disease states such as atherosclerosis [149] and certain cancers [62, 149]. Moreover, it has been reported to induce secretion of i) IL-6 by airway smooth muscle cells [3], ii) IL-8 by bronchial epithelial cells [27] and iii) IL-1 $\beta$  and TNF- $\alpha$  by macrophages [81]. However, S1P can also inhibit IL-12 and increase IL-10 production in maturing dendritic cells activated with microbial products, and hence favors a shift towards a Th2-mediated immune response [64]. Furthermore, S1P and other S1P receptor agonists have recently been described to induce lymphopenia in blood and thoracic duct lymph by sequestration of lymphocytes in lymph nodes, suggesting its potential role in immunosuppressive therapy [88]. The results reported herein show that MTB-infected S1P-treated mice had significant reduction of mycobacterial burden and granulomatous reaction, with prevalent macrophage infiltrate and no evidences of necrotic areas, suggesting that S1P can reduce tissue-damaging T cell-mediated immune response and simultaneously increase the local innate antimicrobial activity exerted by macrophages. In this context, mouse macrophages have been reported to express EDG1 and EDG5 S1P receptors [81], suggesting a S1P involvement in regulation of pulmonary inflammatory responses. Finally, S1P has also been reported to be an important angiogenic factor in serum [39] and an enhancer of endothelial barrier integrity [119]. These evidences may have important implications, as tissue damaging immune response is also associated with the lost of endothelial barrier integrity which may be in turn responsible for the haematogenous dissemination of mycobacteria.

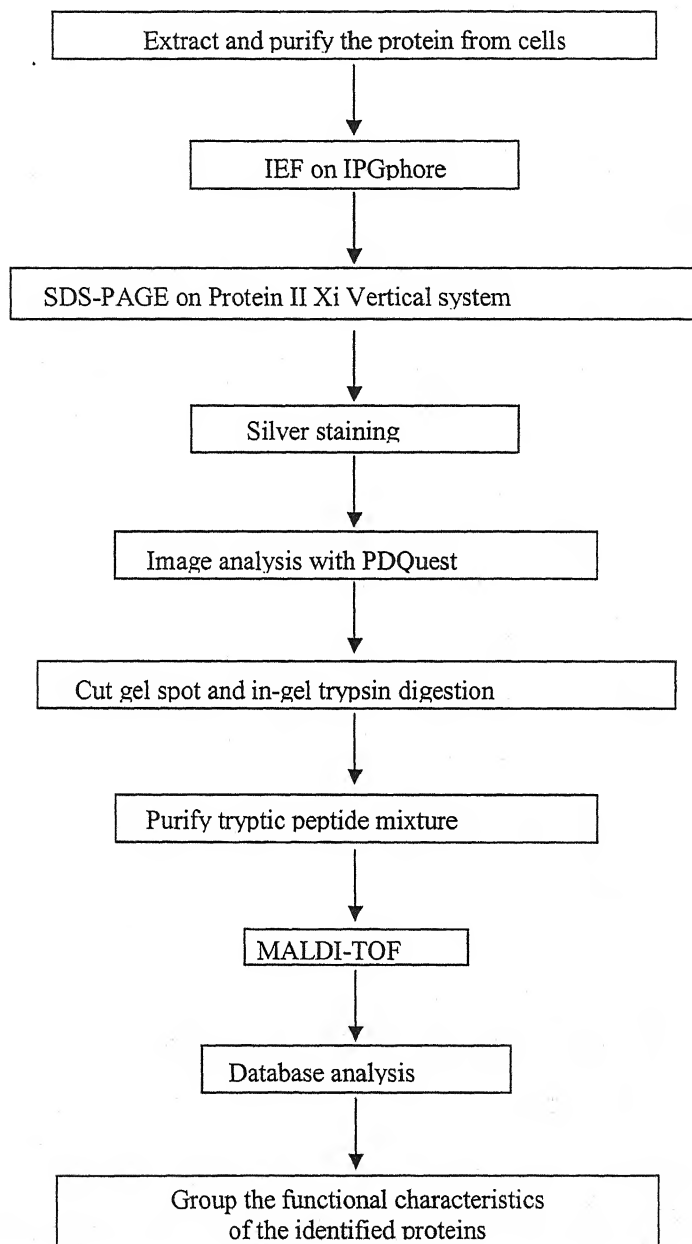
One of the puzzling aspects of tuberculosis is that although one third of world population is infected, fewer than 10% of them develop into active disease during the first few



years following exposure [75] suggesting that components of natural immune response may play an important role in the protection. Moreover, there are strong evidences that primary malnutrition raises the incidence and exacerbates clinical manifestations of tuberculosis [18]. Within this context, the requirement of S1P in the control of primary MTB infection can be hypothesized. Studies with animal models have shown that feeding mice with sphingolipid inhibits colon carcinogenesis [120] and reduce atherosclerosis risk by decreasing plasma cholesterol [65], suggesting that sphingolipids may represent a “functional” constituent of food [144]. On these grounds, it is suggestive to hypothesize a nutritional value of either S1P or its precursors in diet, which can contribute to the antimicrobial immune surveillance of the host and hence to prevent active disease among the third world population harbouring latent MTB.

In order to obtain a holistic view of host biochemical and molecular changes involved in S1P induced macrophage anti-mycobacterial response, we studied protein modulation in the proteome of dTHP1 cells with/out MTB and with/out S1P during *in vitro* culture. An appropriate sample preparation and good 2-DE reference map is absolutely essential for a comparative proteomics studies. Therefore, cells were lysed directly into urea and chaps containing lysis solution in the ice, thus ommiting the activation of proteases. However, protease inhibitors were also included in the lysis buffer in order to prevent the further proteolytic activity of protease inhibitor in subsequent steps. Moreover, in order to remove the nucleic acids and other small ionic salts contaminants protein samples were purified using 2D clean up kit. For the 2-DE system, a commercial IPG strips provided a high level of reproducibility in the IEF direction. The focusing effect of IEF with a nonlinear IPG strips pH 3-10 is superior to that of a linear IPG strips pH 3-10. The vertical SDS-PAGE system provides a higher reproducibility and wider linear dynamic range than the horizontal system [159]. Therefore, for this current study, a combination of an 18 cm IPG strip pH 3-10 NL, a large formate of SDS gel, and approximately 30  $\mu$ g of total protein provided a general profile of the distribution of a macrophage proteome. 2-DE combined with 2D image analysis allowed for the detection of different 21 proteins that were upregulated in course of S1P stimulation. Following staining

protein spots were excised, digested with trypsin, acquired on MALDI-TOF and analysed on online database in order to indentify the protein. However, scheme of whole proteomic protocol could be summarized in the below given **flow chart (Figure 28)**

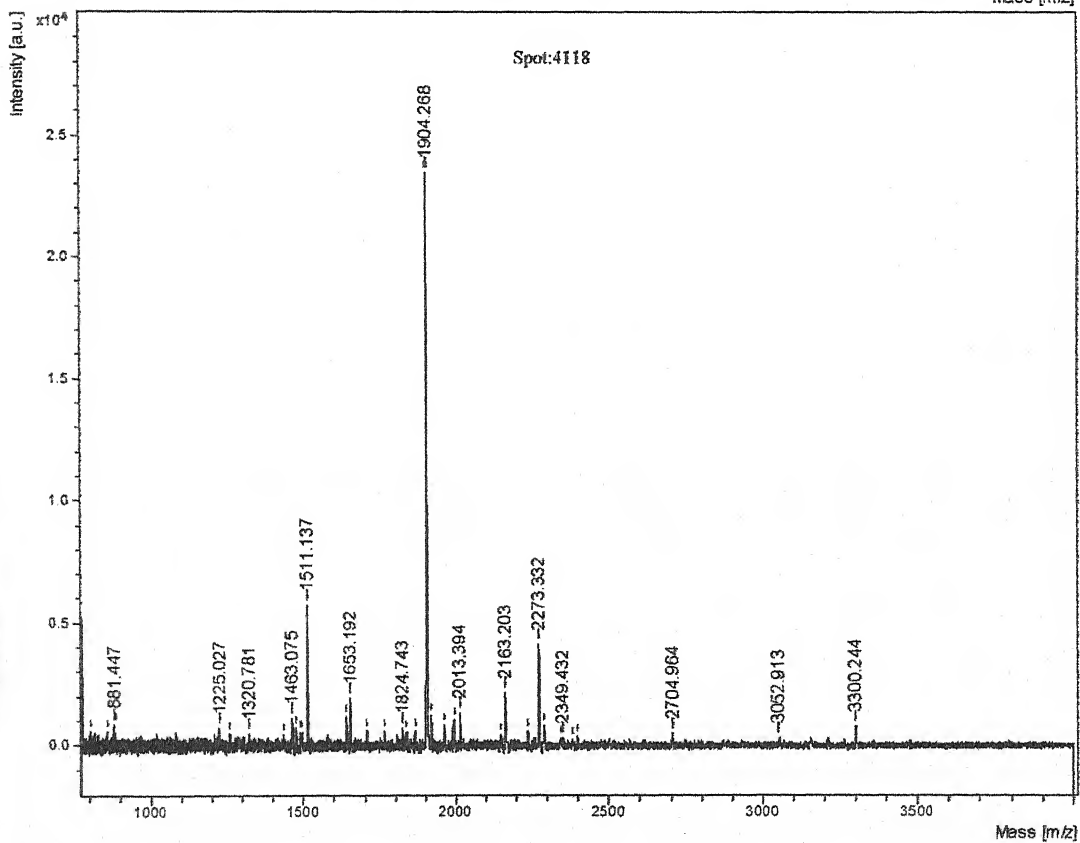
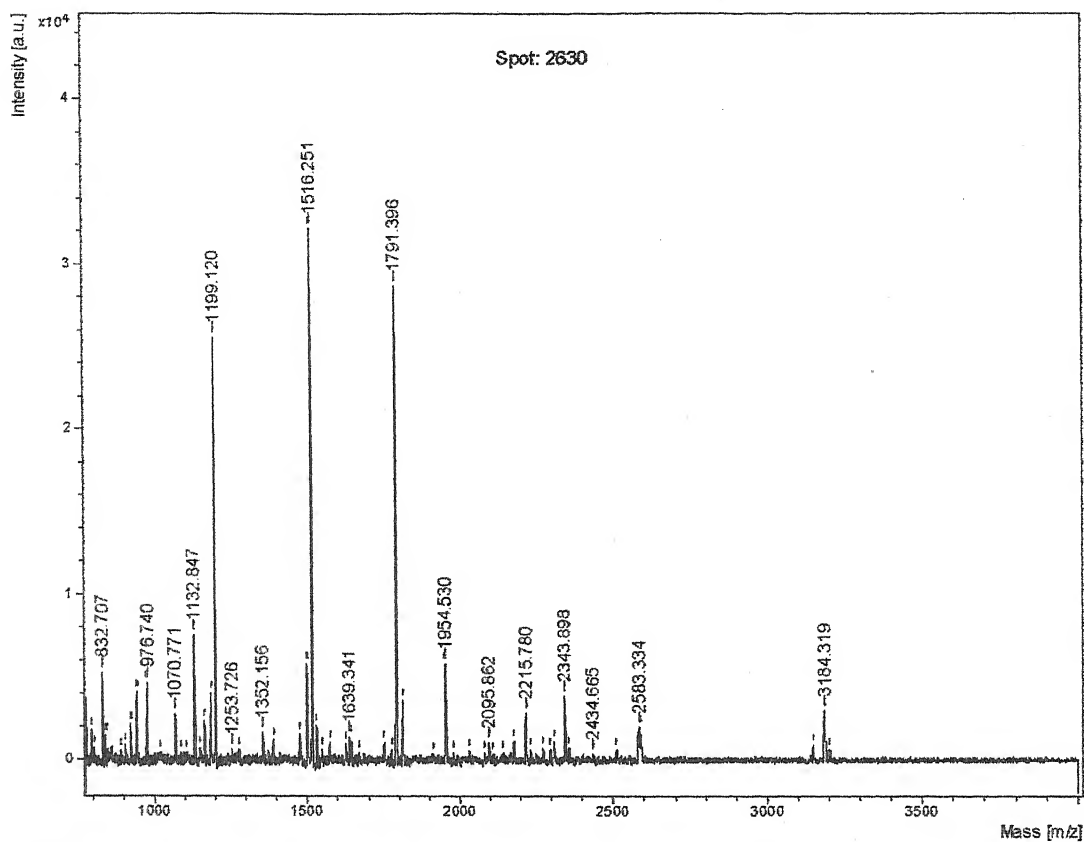


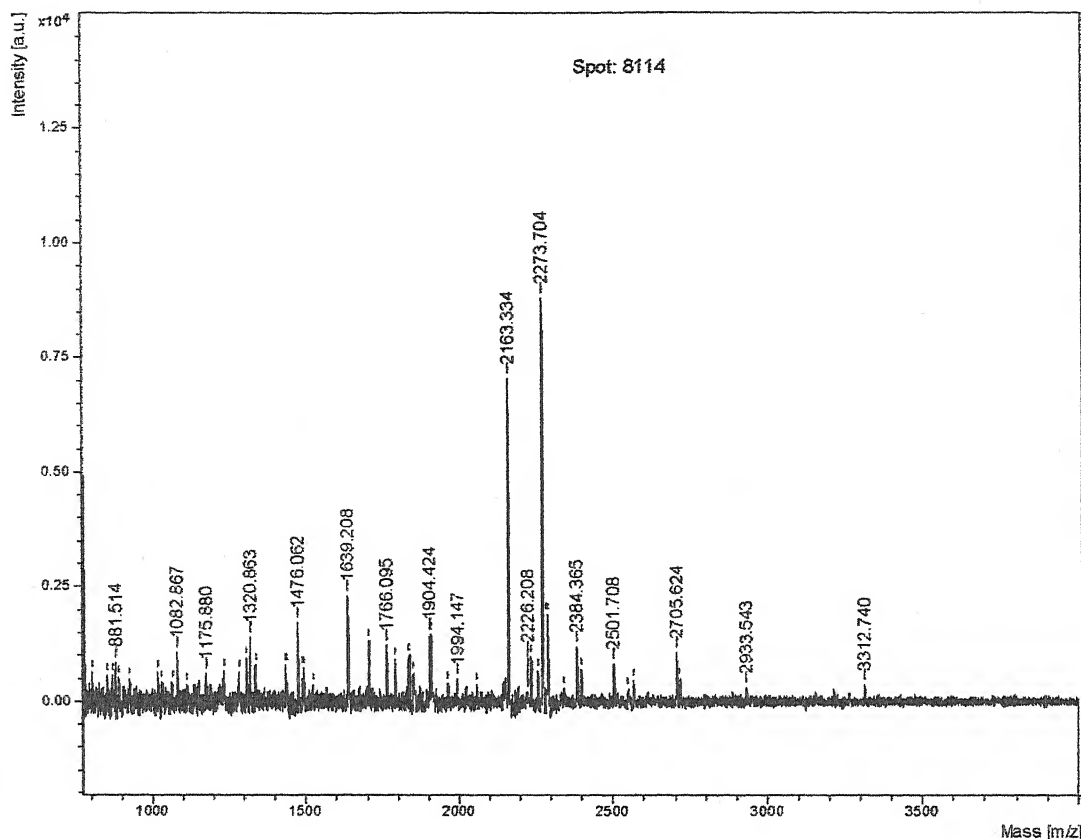
**Figure 28. Overall scheme of the experiments used to establish the reference map of dTHP1 cells**

Spot number 2630 was identified as human  $\beta$  actin protein which was upregulated significantly in presence of S1P stimulation (SWISS PROT accession number: P60709; ACTB\_HUMAN).

Actins are highly conserved proteins that are involved in various types of cell motility and are ubiquitously in all eukaryotic cells. In vertebrates 3 main groups of actin isoforms,  $\alpha$ ,  $\beta$  and  $\gamma$  have been identified. The  $\alpha$  actin are found in muscle tissues and are a major constituents of the contractile apparatus, whereas  $\beta$  and  $\gamma$  actins coexists in most cells types including macrophages as components of the cytoskeleton and a mediators of internal cell motility. Actin assembly has also long been linked to processes of membrane fusion. Recently, it has been shown that some selected lipids, including sphingosine-1 phosphate, activate actin assembly which may be associated with acidification of mycobacteria containing phagosomes resulting in turn in killing of intracellular bacilli, suggesting a role of actine in phagolysosome biogenesis [4]. Based on their work Jahraus A *et al* suggest that unidirectional growth of actin filaments from the phagosomal membrane provides tracks for poteintial fusion partner organelles, such as lysosomes, to move towards the membrane-associated actin plus ends [67]. This model postulates that effectors which stimulate actin assembly by phagosomes may stimulate fusion with late endocytic organelles. In this context, it is possible to hypotheise that S1P upregulated actin proteins integrate into the phagosomal membrane and switch on specific signaling networks that have been turned off by proteins or lipids released by the live pathogen. However, given the complexicity of signaling networks that operate during macrophages activation, more studies are required to distinguish between direct effect of the lipids on phagosomes and on marophages activation in general.

Spot number 8114 was identified as MHC class II antigen with 85.2% sequence coverage. (SWISSPROT accession number Q8SNCO; MHC class II antigen fragment) and was found to be over expressed after S1P treatment. Whereas, in absence of S1P, dTHP cells after infection with MTB do not show any detectable spot of MHC class II fragment protein. As several reports have provided data that MTB infection inhibits MHC Class II presentation of antigens to CD4<sup>+</sup>T cells by the down-regulation of cell surface expression of MHC Class II molecules, which





**Figure 29 . Representative MALDI-TOF spectrum of spots 2630, 4118, 8114.**

**A.** Spot number 2630 has been identified as human actin  $\beta$  protein. (SWISSPROT accession number; P60709, entry name ACTB\_HUMAN).

**B.** Spot number 4118 has been identified as Integrin,  $\beta$ -like 1 with EGF-like repeat domains. (SWISSPROT accession number: Q96QX7; Integrin, beta-like 1 (With EGF-like repeat domains; Fragment)).

**C.** Spot number 8114 has been identified as MHC class II antigen (fragment). (SWISSPROT accession number Q8SNCO; MHC class II antigen fragment).

would contribute to the inability of the host to eliminate a persistent infection. This inhibition of MHC-II function was dependant on TLR-2 and required prolonged exposure to the 19-kDa-lipoprotein [101]. Macrophages infected with MTB also produce cytokines, such as TGF- $\beta$ , IL-10 or IL-6, which can reduce T-cell stimulation [47]. Macrophage impaired the one of the MTB survival mechanism by over expressing the MHC class II antigen after SIP treatment. In this

context the role of MHC class II presentation and CD4<sup>+</sup> cells against MTB control have already accepted. MTB resides primarily in a vacuole within the macrophage resulting in MHC Class II presentation of mycobacterial antigens to CD4<sup>+</sup> T cells. Studies in mouse models deficient in CD4<sup>+</sup> T cells demonstrated clearly that the CD4<sup>+</sup> T-cell subset is required for the control of infection [15]. In addition, other studies demonstrated that adoptive transfer of CD4<sup>+</sup> T cells enhanced protection against TB in mice [104]. The HIV epidemic has demonstrated that the loss of CD4<sup>+</sup> T cells greatly increases susceptibility of human hosts to both acute and reactivation of TB, as HIV<sup>+</sup>PPD<sup>+</sup> subjects have 8–10% annual risk of developing active TB, compared to a 10% lifetime risk for PPD<sup>+</sup>HIV<sup>-</sup> patients [122]. The primary effector function of CD4<sup>+</sup> T cells is believed to be production of IFN- $\gamma$ , and possibly other cytokines, sufficient to activate macrophages, which can then control or eliminate intracellular organisms. The possible other roles of CD4<sup>+</sup> T cells include apoptosis, which has been suggested to be important in controlling MTB infection, conditioning of APCs, help -for B cells and -for CD8<sup>+</sup> T cells, and production of other cytokines. Together with the present finding, this over expression of MHC class II protein after S1P treatment suggests that S1P not only induces the innate immunity of macrophages but also may favors the positive signalling for adaptive immunity against intracellular microbes.

Database analysis revealed the spot number 4118 as a fragment of Integrin,  $\beta$ -like 1 with EGF-like repeat domains [SWISSPROT accession number: Q96QX7; Integrin, beta-like 1 (With EGF-like repeat domains; Fragment)]. Integrin receptors on human neutrophils have been shown to mediate adhesion and phagocytosis, which was shown to dependant on PLD activity. As the initial interaction with surface receptors and mode of phagocytosis influences the subsequent fate of MTB within the macrophages, it is possible to hypothesise that S1P induced PLD activation may lead to activation of Integrin,  $\beta$ -like 1 protein, which may take part into efficient phagocytosis of virulent bacilli, generating essential second messengers required for bacterial killing. However, the signal transduction pathway mediating phagocytosis is the subject of intense investigation and is known to include phospholipases, PKC, protein tyrosin kinase, GTP binding proteins, actin polymerization, and membrane movement.

intense investigation and is known to include phospholipases, PKC, protein tyrosin kinase, GTP binding proteins, actin polymerization, and membrane movement.

As we have shown the importance of host PLD activity in the antimycobacterial response, which was not directly related to its expression level, it is possible to hypothesize that S1P elevated PLD activity act as an important signaling mechanism that may provide a possible downstream signalling to express the effector molecules hence effector mechanisms. Moreover, the roles of these S1P upregulated peptides in antimycobacterial effector mechanism are need to be confirmed through RNA interference (iRNA) assay.

## Summary and conclusions

Altogether, the essence of present study describes:

1. The identification of a second lipid messenger able to induce antimycobacterial activity in macrophages.

1. The identification of an additional antimicrobial property (*in vitro*, *in vivo*, *ex vivo*) of a second lipid messenger without any possible interference with macrophage viability;

2. The molecular mechanisms underlying such antimicobacterial activity involving host phospholipase D which promotes phagosome-lysosome fusion;

3. The confirmation of *in vitro* results, in a mouse model of MTB infection, where present study show about 50% decrease of mycobacterial burden both in the lung and in the spleen of infected mice. Histology analysis revealed a strong reduction of pulmonary tissue damage and improvement of the number of functional parenchyma on lung surface in comparison to control. These results may have very important implications in clinical point of view, as very few study have been reported to confirm, the observed *in vitro* antimicobacterial activity, in an *in vivo* model of infection and such compound may be used as aerosolic therapy (studies are actually in progress to better define the therapeutical possibility of this compound).

4. Proteomics analysis as a powerful tool to analyse the host protein modulation in course of active MTB infection can yields insights into complementary mechanisms of mycobactericidal activity exerted by activated macrophages.

In this context, my present work suggests PLD as a potential molecular target for innovative immunotherapeutic approaches aiming at promoting natural immunity to tuberculosis. In addition, the introduction of S1P as a tuberculocidal agent may yield an insight to enhance the natural immunity of macrophages. Moreover, further characterization of the deep mechanism by which S1P-induced PLD promotes killing of MTB within infected human macrophages may aid



to design and implementation of effective therapeutics against tuberculosis. Finally, to this regards a combined effort on genome, transcriptome and proteome, in a unified policy, can offer good prospects for the development of an efficacious therapeutic intervention and candidate vaccines.

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## List of publications

- 1) R.P.Tiwari, **Sanjay K Garg**, Dileep Tiwari, P Ravindranath Tagore, Ramesh Chandra, Rucha Karnik, Nirav Desai, Nimesh Thaker, Seema Bhatt, P.K.Ghosh, Ramnani V, Mrs. Q. Khan, Maurizio Fraziano, Vittorio Colizzi and P.S. Bisen. Development Of A Simple And Reliable Diagnostic Test For Menengial, Pulmonary And Extrapulmonary Tuberculosis (*J. Clin. Microbiol., Revised*).
- 2) **Sanjay K. Garg**, Marilina B. Santucci, Segrouchni Fuoad, Cesare Saltini, Prakash S. Bisen, Vittorio Colizzi, Maurizio Fraziano. Tuberculosis Therapeutics: Past achievements, present road blocks and future prospectives (*Letters in Drug Design and Discovery; In press*).
- 3) Marilina B. Santucci, Maria L. Bocchino, **Sanjay K. Garg**, Vittorio Colizzi, Cesare Saltini, Maurizio Fraziano. The proportion of pulmonary CCR5+ CD4+ T lymphocytes is increased in the course of active tuberculosis. (*European respiratory journal; In press*).
- 4) Antonio Ciaramella, Anna Cavone, Marilina B. Santucci, **Sanjay K. Garg**, Nunzia Sanarico, Marialuisa Bocchino, Domenico Galati, Angelo Martino, Giovanni Auricchio, Melania D'Orazio, Graham R. Stewart, Olivier Neyrolles, Douglas B. Young, Vittorio Colizzi, Maurizio Fraziano. Cell wall associated 19-kDa lipoprotein induces apoptosis and IL-1 $\beta$  release in the course of mycobacterial infection. (*J. Infect. Dis.; In press*)
- 5) **Sanjay K. Garg**, Elisabetta Volpe, Graziana Palmieri, Maurizio Mattei, Domenico Galati, Angelo Martino, Maria S. Piccioni, Emanuela Valente, Elena Bonanno, Paolo De Vito, Patrizia M. Baldini, Luigi G. Spagnoli, Vittorio Colizzi, Maurizio Fraziano. Sphingosine 1-phosphate

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6) **Sanjay K. Garg**, Giovanni Auricchio, Angelo Martino, Elisabetta Volpe, Antonio Ciaramella, Paolo De Vito, Patrizia M. Baldini, Vittorio Colizzi, Maurizio Fraziano. Role of Macrophage phospholipase D in natural and CpG-induced anti-mycobacterial activity. *Cell Microbiol.* 2003 Dec;5(12):913-20.

7) Prakash S. Bisen, **Sanjay K. Garg**, P. Ravindra Nath Tagore, Ram P. Tiwari, Ramesh Chandra, Rucha Karnik, Nimesh Thaker, Nirav Dasai, P. K. Ghosh, Maurizio Fraziano, Vittorio Colizzi. Analysis of Shotgun Expression Library Of *Mycobacterium tuberculosis* Genome For Immunodominant Polypeptides: Potential Use In Serodiagnosis. *Clin Diagn Lab Immunol.* 2003 Nov;10(6):1051-8.

8) **Sanjay K. Garg**, R.P. Tiwari, Dileep Tiwari, Rupinder Singh, Dolly Malhotra, V. K. Ramnani, GBSK Prasad, Ramesh Chandra, Maurizio Fraziano, Vittorio Colizzi, Prakash S. Bisen. Diagnosis of Tuberculosis: Available technologies, Limitations and Possibilities. *J Clin Lab Anal.* 2003; 17(5):155-63.